THE EFFECT OF L-ALANYL-L-GLUTAMINE DIPEPTIDE ON PROTEIN TURNOVER IN HEALTHY POSTABSORPTIVE MAN AND IN PATIENTS UNDERGOING MAJOR ELECTIVE SURGERY

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SUMMARY

Skeletal muscle plays a key role in the metabolic response to injury and sepsis and in the negative nitrogen balance often associated with these conditions. The intra-muscular concentration of glutamine, a "non-essential" amino acid, shows a marked fall as a result of trauma or surgery and a close relationship has been demonstrated between muscle protein synthesis and breakdown, and the intramuscular concentration of glutamine. Provision of extra glutamine to the post-operative patient as the dipeptide L-Alanyl-L-Glutamine (Ala-Gln) results in improved whole body nitrogen balance. In the present studies the acute effects and the possible mechanisms of action of this dipeptide on muscle protein balance have been studied in 15 healthy post-absorptive subjects and in 16 intensive care unit patients who had undergone major elective abdominal surgery.

Whole body and skeletal muscle protein turnover were investigated using primed, constant infusions of the stable isotope tracers L-[1-^{13}C]leucine and L-[^{15}N]phenylalanine, with determination of plasma leucine-C flux and $^{13}CO_2$ production, assay of L-[1-^{13}C]leucine incorporation into mixed anterior tibial muscle protein sampled by biopsy and arteriovenous forearm exchange of L-[^{15}N]phenylalanine with net release of 3-methylhistidine from forearm tissues to assess muscle mixed protein turnover and myofibrillar protein breakdown respectively.

Parenteral administration of the dipeptide Ala-Gln increased plasma and intramuscular glutamine concentrations in healthy subjects and in post-operative patients. In healthy postabsorptive subjects, mixed muscle protein synthesis was increased with no significant effect on mixed muscle protein breakdown or on

whole-body protein turnover, although small reductions were observed in whole-body flux of leucine and phenylalanine. The increased glutamine availability in muscle *per se* appeared to stimulate muscle protein synthesis, calculated on the basis of the enrichments of plasma α-ketoisocaproate. Since Ala-Gln infusion does not appear to alter whole-body protein turnover it is likely that in the absence of availability of dietary amino acids, skeletal muscle protein synthesis is increased by glutamine at the expense of non-muscle protein, as the muscle free amino acid pool could not sustain such an increase for more than a few minutes.

In post-operative patients receiving a standard non-glutamine containing amino acid mixture used in conventional total parenteral nutrition (TPN), short-term Ala-Gln supplementation acutely increased mixed muscle protein synthesis, as determined by L-[1-^{13}C]leucine incorporation into M. tibialis anterior and by L-[^{15}N]phenylalanine arteriovenous forearm exchange, although the absolute values observed for protein synthesis were lower than in the post-absorptive healthy subjects. There was no significant effect of Ala-Gln dipeptide supplementation on muscle protein breakdown, or whole-body kinetics although the whole-body flux of both leucine and phenylalanine were slightly lower with Ala-Gln supplementation. Albumin synthesis was increased by Ala-Gln supplementation.

These results suggest that increased glutamine provision, as the dipeptide Ala-Gln, has a small anabolic effect on skeletal muscle in healthy post-absorptive man. In patients undergoing major elective surgery, short-term glutamine dipeptide supplementation of conventional TPN results in a marked acute anabolic effect on skeletal muscle by stimulating protein synthesis, without affecting breakdown.

DECLARATION

The work described in this thesis was entirely undertaken by myself with the following specific exceptions:

1. The gas chromatography mass spectrometers and the isotope ratio mass spectrometry were programmed by Dr. K. Smith and Dr. C. Scrimgeour.
2. Amino acid concentrations in extracts from plasma and intramuscular water were determined by Mr. B. Weryk.
3. Analyses of hormone concentrations in plasma were performed by the department of Biochemistry, Ninewells Hospital and Medical School.
4. Albumin extraction from plasma and determination of its isotoope enrichment was performed by Mr. S. Downey.
5. Assistance was obtained from Dr. K. Smith and Mr. S. Downey in preparation of samples for gas chromatography mass spectrometry.

I would like to dedicate this thesis to my parents Dr. Rajendra Barua and Mrs. Latika Barua and to my wife Urbashi.

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ABBREVIATIONS

ACTH	Adenocorticotrophic hormone
ADH	Anti-diuretic hormone
Ala-Gln	L-alanyl-L-glutamine dipeptide
Ala+Gly	L-alanine and glycine
APE	Atoms percent excess
BCAA	Branched chain amino acids
CI	Confidence interval
EDTA	Ethylenediamine-tetra acetic acid
EGA	Ethylene glycol adipate
GC-MS	Gas chromatography-mass spectrometry
GH	Growth hormone
Gly-Tyr	Glycyl-L-tyrosine dipeptide
IGF-1	Insulin like growth factor
IRMS	Isotope ratio mass spectrometry
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
IMW	Intramuscular water
KCl	Potassium chloride
α-KIC	α-ketoisocaproic acid
kJ	Kilojoules
KOH	Potassium hydroxide
Li-Hep	Lithium heparin
MTBSTFA	*N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide
m/z	Mass to charge ratio
NaOH	Sodium hydroxide
PCA	Perchloric acid
t-BDMS	*tertiary*-butyldimethylsilyl
TCA	Trichloroacetic acid
SEM	Standard error of the mean
SDS	Sodium dodecyl sulphate
TNF	Tumour necrosis factor
TPN	Total parenteral nutrition
tRNA	Transfer ribonucleic acid

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INTRODUCTION

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1.1 Early Observations of Injury in Man

The danger of injury, either of an accidental nature or that inflicted deliberately, has always been present in man's environment and methods for treating the wounded probably have a history as long as that of mankind. However, attempts to understand the mechanisms by which critical illness produced tissue loss were only begun in the nineteenth century and detailed investigations were not undertaken till the third decade of the present century. In 1887 Bernard (Burns, 1988) noted that haemorrhage caused a rise in blood sugar, and shortly after this in 1899 Crile (Burns, 1988) published an experimental study of shock, in which for the first time, arterial vasoconstriction and consequent reduced tissue perfusion was identified as a common feature of injury. Evidence to link the circulatory abnormality with a metabolic lesion was produced by Cannon and Bayliss in 1919 (Burns, 1988) who used the Van Slyke apparatus to show that fixed acids (such as lactic acid) accumulated in the blood of soldiers with low blood pressure. In 1918 Dale and Richards (Burns, 1988) were studying the effects of tourniquet removal from injured limbs. They noted an improved survival if amputation was carried out before the tourniquet was removed and they concluded from this that a major factor in injury was the release of toxic materials into the systemic circulation from the wound.

Although increased losses of urinary nitrogen in man had been observed during disease and following trauma by Vogel in 1884 and by Malcolm in 1893, respectively (Clague, 1981), the implication of this loss for protein metabolism only became apparent in the nineteen thirties. Cuthbertson (1932), from simple observations and careful analyses on injured individuals, was able to characterise

the major sources and composition of this nitrogen loss. He and his colleagues found that the metabolic response to injury appeared to be subdivided into two phases, described as 'ebb' and 'flow', the latter being associated with increased metabolism and the net loss of nitrogen from the body (Cuthbertson, 1942). This loss appeared to be related to the severity of the injury (Cuthbertson, 1932), the age of the patient (Cuthbertson, 1932), the pre-injury nutritional status of the individual (Calloway *et al.*, 1955) and nutritional intake following trauma (Cuthbertson, 1936). Administration of extracts of endocrine glands (Cuthbertson *et al.*, 1942) or hormonal involvement (Johnston, 1967) could also modify the nitrogen loss, which was thought to arise from increased muscle protein catabolism (Cuthbertson, 1930). The metabolic response to trauma and the factors which modulate its extent are discussed later in this chapter.

1.2 Early observations on the relationship between injury and nutrition

In 1936, Studley described the effect of antecedent malnutrition upon the outcome of (surgical) injury: in patients undergoing surgery for peptic ulcer, those who had lost a substantial amount of weight pre-operatively had a much higher mortality than those of normal weight (Allison, 1986). In 1945 Munro and Chalmers (Munro and Chalmers, 1945) published a study on rats, describing the metabolic response to fracture at different levels of protein intake. The animals were divided into three groups: one group was given a high protein intake; a second group was given a low protein intake and a third group was given a protein-free diet for 12 days before being subjected to fracture of the hind limb. The rise in

nitrogen excretion post injury was less as protein intake fell. That this difference depended upon the food intake before the fracture was shown by a second study in which the low and high protein intake groups were switched at the time of fracture. Their nitrogen excretion still did not reach that of the group which had been given the high protein intake throughout.

These early observations have provided insight into the relationship between injury and nutritional state. More recent research has been directed towards three major issues: firstly, variations in the metabolic response under different conditions and identification of the mediators which control the metabolic response; secondly, a better understanding of the abnormal protein metabolism seen after injury with exploration of the possibilities for its modulation if this were demonstrated to be desirable; thirdly, a clearer understanding of the type and quantity of energy required by stressed individuals. Consideration of the first and second of these areas forms the basis for the remaining part of this chapter.

1.3 The metabolic response to trauma

1.3.1 Introduction

Of the two main phases of the metabolic response to injury (Cuthbertson, 1930; Table 1.1) the early *ebb* phase is characterised by hypovolaemia and subsequent sympathetic and adrenal response, whilst during the later *flow* phase the injury results in loss of protein at an accelerated rate.

1.3.1.1 The ebb phase

Hypovolaemia, characterised in the clinical situation by a patient who is pale, clammy and tachycardic, plays a major role in the ebb phase which lasts until circulating volume is restored. The importance of the brain in the generation of the early endocrine response to injury has been demonstrated by Hume and Egdahl

Table 1.1. Metabolic response to Injury - ebb and flow phases, (after Cuthbertson).

Ebb phase	Flow phase
Hypometabolic	Hypermetabolic
Decreased energy expenditure	Increased energy expenditure
Extremities cold and clammy	extremities warm
Cardiac output reduced	Cardiac output increased
Low core temperature	Elevated core temperature
Normal glucose production	Increased glucose production
Elevated blood glucose	Blood glucose normal or slightly elevated
Elevated circulating catecholamines	Circulating catecholamines high normal or elevated
Glucagon elevated	Glucagon elevated
Low insulin concentration	Insulin concentration low or elevated
Mediated by central nervous system	Mediated by central nervous system and cytokines

Adapted from Hill (Hill, 1992)

(Hume and Egdahl, 1959). In an experiment in which they disconnected a limb from the body of an anaesthetised animal, leaving only the sciatic nerve and the femoral vessels, the level of corticosteroids in adrenal venous blood was found to rise immediately in response to a burn injury to the isolated limb. This response did not occur when the sciatic nerve was divided. Hume and Egdahl also demonstrated that the early hormonal response to injury did not occur unless the central nervous system was intact. Thus pain, hypovolaemia, acidosis and hypoxia initiate the neural afferent signals to the brain and after processing in the hypothalamus, leads to increased activity of the sympatho-adrenal system which is accompanied by release of adrenocorticotrophic hormone (ACTH) and growth hormone (GH) from the anterior pituitary and anti-diuretic hormone (ADH) from the posterior pituitary. ACTH release results in a rise in plasma cortisol and the renin-angiotensin system is activated (Hill, 1992). The resulting vasoconstriction decreases renal blood flow, glomerular filtration rate, sodium excretion (hence sodium and water retention) and urine production. Fluid retention extends beyond the ebb phase but diuresis occurs within the first 72 hours in most cases. Plasma insulin levels may be variable but they are relatively low in relation to the usually high glucose concentration.

1.3.1.2 The flow phase

The duration of the flow phase is proportional to the severity of the injury and it is followed by a third, anabolic, phase during which the protein and energy stores lost in the early post-injury period begin to be repleted. The flow phase is

characterised by degradation of muscle protein to release amino acids such as glutamine and alanine. The later amino acid in particular undergoes de-amination and through the process of gluconeogenesis supplies glucose which is essential for the brain and healing tissues in particular. In severely injured patients and in severe sepsis the flow phase is associated with an increased metabolic rate but the rise is small or negligible in patients undergoing elective surgical procedures (Hill, 1992). The patient's energy requirements during this phase are mostly met by fat oxidation. The plasma concentrations of the counter regulatory stress hormones glucagon, catecholamines and cortisol fall during this period. The plasma insulin concentration rises during the flow phase to reach its maximum value. Paradoxically loss of lean tissue is also maximal at this time and the apparent resistance to the usual anabolic effects of insulin are poorly understood.

1.3.2 Cytokines and the metabolic response to trauma

Recent studies have highlighted the possible role of cytokines or cell mediators released from the site of the wound (Fong and Lowry, 1990). These may have local as well as systemic effects. Interleukin-1, interleukin-2, interleukin-6, tumour necrosis factor (TNF) and interferon γ are important mediators of the integrated metabolic response (Cruickshank *et al.*, 1990). At least two of these cytokines, TNF and interleukin-2, are post-inflammatory circulating signals that are probably involved in the metabolic response in serious sepsis. Endotoxin, a lipopolysaccharide found on the cell wall of gram-negative bacteria, is a potent stimulus for the production of TNF. TNF infusion replicates many of the clinical

and metabolic features of sepsis, including fever, hypotension, anorexia, hyperglycaemia and a negative nitrogen balance (Tracey *et al.*, 1986; Michie *et al.*, 1988a, 1988b, 1988c and 1990). Its effects also include muscle proteolysis, increased amino acid uptake by the liver and increased lipolysis. Interleukin-1 has been called proteolysis inducing factor and also is a principal inducer of acute phase protein synthesis. Interleukin-2 and interleukin-6, in addition to their metabolic effects are also stimulators of the immune system. Interleukin-2 is a growth factor for activated T cells and it activates cytotoxic T cells. Interleukin-6 plays a role in the differentiation of B cells into plasma cells, T cell activation and is a growth factor for eosinophils (Flechner, 1994).

1.4 Neuroendocrine Responses to Injury

1.4.1 The Afferent Limb of the Neuroendocrine Response to Injury

It is well established that the neuroendocrine response to sepsis and trauma effects many of the observed changes in metabolism (Figure 1.1). Trauma in all its forms may result in activation of a number of different afferent pathways. The neuroendocrine response to trauma can be blocked by division of the nerves to the area being traumatised. Conscious perception of pain is not necessary for this neuroendocrine response but the central nervous connections to the hypothalamus are essential (Hume and Egdahl, 1959). Although reception of pain at the site of injury and afferent impulses related to changes in effective circulating blood volume play the overriding roles in the early neuroendocrine response to trauma, as injury

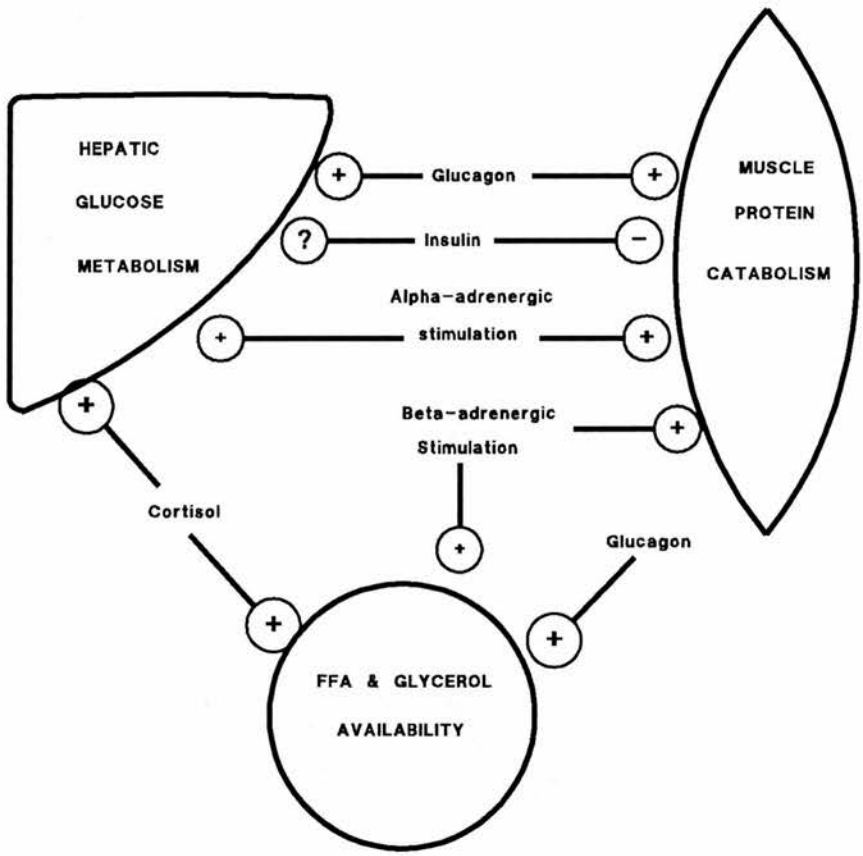

Figure 1.1 An overview of the important hormonal regulatory factors in the regulation of metabolism in septic and injured patients. FFA = free fatty acids. (Adapted from Douglas and Shaw, 1989)

progresses, other stimuli such as acidosis, hypoxia, hypercapnia, changes in body temperature as well as the release of local factors from injured tissues may all contribute to the overall response.

1.4.2 The Efferent Limb of the Neuroendocrine Response to Injury

The best known neuroendocrine response to injury is the increase in ACTH secretion and the resultant increase in adrenocortical secretion of glucocorticoid hormones (Ganong, 1986). The mechanism by which increased plasma glucocorticoids exert a protective effect is still poorly understood, although the increase is clearly important, for in its absence the level of tolerance to stress is reduced and relatively mild noxious stimuli can lead to death (Ganong, 1983a, 1983b). Sympathetic output is also increased by injury resulting in stimulation of an increased output of adrenal medullary hormones. It has been postulated that one of the reasons why the increase in glucocorticoid secretion can be life-preserving is that it maintains the responsiveness of vascular smooth muscle to the constrictor action of catecholamines (Ramey and Goldstein, 1957). This hypothesis has not been proven however. Stressful stimuli, in particular any significant decrease in extracellular fluid volume, also stimulate the secretion of vasopressin (Ganong, 1983a, 1983b). Vasopressin contributes to the maintenance of extracellular fluid volume by retaining water although this contribution is modest since up to two-thirds of the retained water enters cells. Vasopressin also produces vasoconstriction. Many stressful stimuli also increase circulating glucagon levels by stimulation of the sympathetic nerves to the pancreas and the β-adrenergic

receptors in the pancreatic islet cells (Unger and Orci, 1981).

1.5 Factors Modifying the Response to Injury

A variety of factors may modify the endocrine-metabolic response to trauma and some have relevance in the clinical situation.

1.5.1 General Anaesthesia

Although general anaesthesia limits the immediate perception of injury, the processing of noxious stimuli to the hypothalamus is not interrupted, thus there is no significant alteration of the stress response (Oyama, 1983).

1.5.2 Afferent Neurogenic Blockade: Regional Anaesthesia

In 1910, Crile (cited by Kehlet and Schulze, 1986) demonstrated that blockage of nociceptive stimuli by a peripheral nerve block may ameliorate the response to injury and further evidence for this was provided in a series of studies by Hume and Egdahl (Hume and Egdahl, 1959) demonstrating the importance of the central nervous system in mediating the adrenocortical response to injury. In recent years a number of studies have shown that blockage of afferent neurogenic stimuli by regional anaesthesia (either spinal or epidural) with local anaesthetic

agents may prevent or attenuate a predominant part of the endocrine-metabolic response to elective surgical trauma particularly for operations performed on the lower part of the body, where adequate afferent block was easier to achieve (Kehlet, 1982 and 1984).

1.5.3 Systemic opiate administration

The clinical use of 'high'-dose opiate administration is limited because of the concomitant depression of the respiratory system. The available data strongly suggest that standard post-operative doses of morphine administered at 4-10 mg every 4-6 hours or other opiates at equivalent doses have no significant effect on the stress response (Kehlet, 1986).

1.5.4 Modulation of Anabolic/Catabolic Hormone Ratio

Since the endocrine response to injury is characterised by increased secretion of catabolically active hormones (catecholamines, cortisol and glucagon) and impaired secretion or effect of anabolically acting hormones (insulin, testosterone) theoretically it would seem possible to counter the net catabolic state by blocking the action of catecholamines by pharmacological blockade or by administration of anabolic hormones.

1.5.5 Alpha-Adrenergic and Beta-Adrenergic Blockade

Administration of the alpha-adrenergic receptor blocker phentolamine has only minor or no effect on various metabolic responses to surgical stress (Kehlet and Schulze, 1986). Beta-adrenergic receptor blockade, however, with propranolol reduced oxygen consumption levels in burns patients (Wilmore *et al.*, 1974) and following surgical stress, propranolol also reduced the trauma-induced increase in plasma glucose, lactate and free fatty acids (Tsuji *et al.*, 1980). However, no studies are available on the influence of adrenergic blockade on the neuro-endocrine response to elective surgery and on post-operative nitrogen balance.

1.5.6 Insulin

Insulin infusion at doses of 35 to 70 mU/kg/h during gynaecological surgery caused decreases in plasma glucose, free fatty acids and beta-hydroxybutyrate and raised blood lactate and growth hormone levels (Hall *et al.*, 1983). Infusion of large doses of insulin in a mixed group of catabolic patients with pronounced urinary nitrogen excretion resulted in a significant reduction in the nitrogen loss (Woolfson *et al.*, 1979).

1.5.7 Growth Hormone

Studies on patients with burn injuries (Wilmore *et al.*, 1974; Soroff *et al.*,

1967) as well as studies on post-operative patients (Ponting, 1988; Ward, 1987) have shown that human growth hormone administration may result in a marked increase in nitrogen retention. The mechanism, however, of this increased nitrogen retention is yet unknown (Cameron *et al.*, 1988) although various studies in both man and in animals have raised the possibility that protein synthesis is increased indirectly with this action being mediated by insulin-like growth factor 1 (IGF-1) (Swenne and Hill, 1989; Bates and Holder, 1988; Schwartz, 1982; Fong *et al.*, 1989).

1.5.8 Substrate Administration

Although the influence of nutritional support can alter several aspects of the response to injury, in general, whereas provision of substrates may improve post-injury catabolism it does not appear to do so to any major degree and the fundamental neuro-endocrine and metabolic responses are not prevented (Kehlet and Schulze, 1986).

1.5.9 Modulating the Response to Severe Injury

When injury is limited, as in uncomplicated elective surgery, the response is slight and of shorter duration. In major accidental trauma, or in elective surgery complicated by sepsis and major haemodynamic alterations, the neuroendocrine response is enhanced and the metabolic effects such as the acute loss of lean tissue mass may be substantial. Recent advances in molecular biology, in recombinant

technology, by providing monoclonal antibodies to endotoxin (Ziegler *et al.*, 1991) and various cytokines such as TNF (Shimamoto *et al.*, 1988), raise the prospect of effective intervention in and modification of the metabolic response to trauma with the possibility of realising significant benefits in the clinical field of patients being treated in intensive therapy units.

1.6 Nitrogen Balance and Protein Turnover

1.6.1 Nitrogen Balance

The chemical measurement of nitrogen in food and excreta allowed Boussingault to conduct detailed nitrogen studies on farm animals in 1842 (Holmes, 1984). It was almost a century later that Cuthbertson and co-workers (Cuthbertson, 1932; Howard *et al.*, 1944) and Moore and Ball (1952) demonstrated that the increase in nitrogen excretion following injury was proportional to the severity of that injury and that most of the extra nitrogen loss was in the form of urea. Although studies of nitrogen balance give information on the net result of protein synthesis and breakdown, the nitrogen balance method cannot be used in assessing whole body protein turnover as no direct information is obtained on the contributory processes of protein synthesis and protein breakdown (Bender, 1985).

1.6.2 Proteins and Amino Acids

In a normal man of 70 kg body weight, body protein accounts for approximately 10-11 kg and of this 1/2 to 2/3 is muscle (Cahill, 1970). Approximately 45% of total body mass is represented by skeletal muscle (which also represents 70% of lean body mass) and this tissue is composed of approximately 17% protein. In skeletal muscle 65% of its protein is in the form of the two proteins of the contractile myofilaments, actin and myosin. Amino acids may be present in skeletal muscle in either in the free amino acid pool or bound as protein (Figure 1.2). Intracellular free amino acids are incorporated into protein by protein synthesis. Amino acids may enter the free pool from the diet, from breakdown of protein or from *de novo* synthesis (of the non-essential amino acids).

Skeletal muscle has an important mechanical role in the maintenance of posture and in movement; it also has a vital metabolic role. Approximately 50% of the body's free amino acid pool exists in skeletal muscle (Munro, 1970), making it the largest reservoir of amino acids in the body. These amino acids are available for a variety of metabolic processes. They may be used as:

1) Substrates for protein synthesis, either locally or in other tissues, and of albumin and acute phase proteins which are important in the body's response following injury.

2) Precursors for gluconeogenesis in maintaining glucose homeostasis (Cahill, 1970; Daniel *et al.*, 1977); alanine and glutamine are the main gluconeogenic amino acids and *via* the glucose-alanine cycle they effectively transport fuel from skeletal muscle to liver where they act as substrates for glucose synthesis (Figure 1.3). (Felig, 1975).

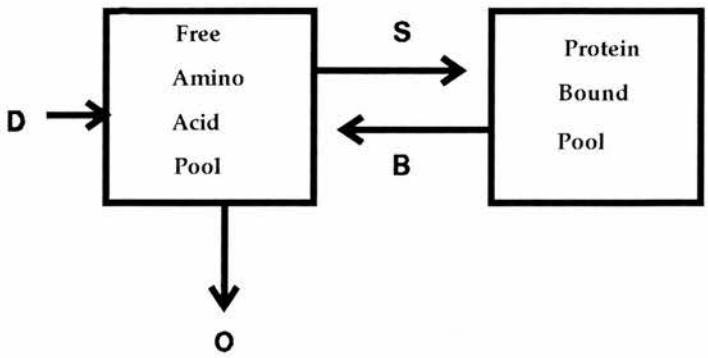

Figure 1.2 Schematic representation of a two pool model of protein turnover. D = diet; S = synthesis; B = breakdown; O = oxidation

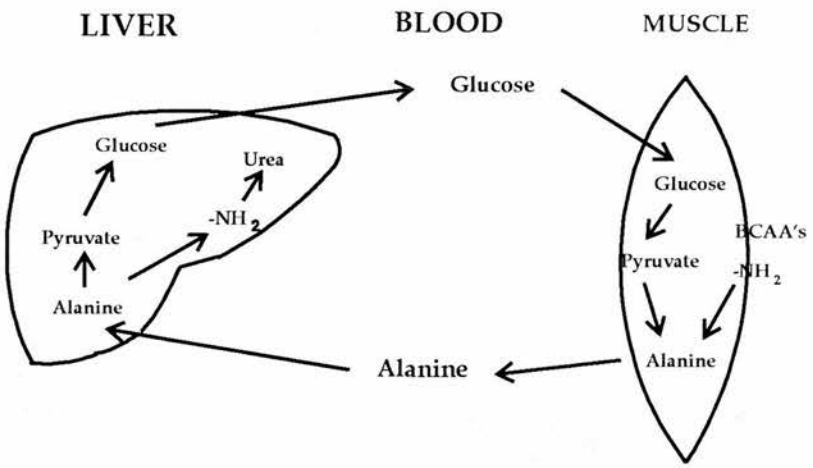

Figure 1.3 Schematic representation of Glucose - Alanine cycle. Adapted from Felig, 1975.

3) Intermediary metabolites of branched chain amino acids which may act as substrates for fatty acid synthesis.

4) Certain amino acids may act as substrate for further intermediary metabolism. Glutamine is used by gut enterocytes in the synthesis of alanine (Matsutaka *et al.*, 1973), in the kidney for ammoniagenesis (Cahill, 1970) and as a fuel for white cells and other rapidly turning over cells..

5) Amino acids may also have a buffering role in acid-based alternative e.g. that occurring in starvation when there is no cation to accompany ketone, bicarbonate, sulphate and phosphate excretion. Glutamine is metabolised by the kidney to glutamate and the ammonium ion is excreted in the urine (Pitts *et al.*, 1965; Golden *et al.*, 1982).

1.6.3 Free Amino Acid Pools and their Significance

Munro (1970) in his review of the role of free amino acids as "the units of currency" of protein metabolism, has commented on the following features of free amino acid pools:

1) Free amino acids account for only a small proportion (estimated at between 0.5% -1.0%) of the total amount of amino acids in the body.

2) Free amino-nitrogen is predominantly available in the form of four of the eight non-essential or dispensable amino acids: alanine, glutamate, glutamine and glycine.

3) Around 50% of the free pool of each amino acid is present in skeletal muscle and indeed of all tissues muscle accounts for the largest proportion of the

body's free pool.

4) The concentrations of the non-essential free amino acids are higher within cells than in plasma and this would suggest that the concentration difference is maintained by a concentration mechanism which would be energy dependent (e.g. glutamine, using the sodium gradient) or binding-dependent (e.g. taurine).

5) The relative amounts of the different free amino acids are unrelated to their relative proportions in the protein bound amino acid pool, as well as to the known requirements for essential amino acids. Branched chain amino acids (BCAA) for example account for 20% of protein composition but only 1% of the free amino acid pool in skeletal muscle.

A number of factors affect the free amino acid pool sizes and these include:

1) Dietary intake, the main factor causing substantial and frequent alterations in the free amino acid pool.

2) Protein turnover, which does not make any net contribution in steady state conditions but may contribute in situations where the rates of protein synthesis and breakdown are not equal in magnitude.

3) Non-essential amino acids are synthesised *de novo*.

4) Transamination, for example of pyruvate to alanine and the amidation of glutamate to glutamine are important in the transport of amino-nitrogen from peripheral tissues (e.g. muscle) to liver (Felig *et al.*, 1969; Felig and Wahren, 1974). It is likely then that changes in the concentrations of these

amino acids may reflect predominantly changes in the overall pattern of amino acid oxidation or of gluconeogenesis.

5) The concentration of amino acids in intracellular pools will be affected by the activity of the transport systems. For most amino acids the intracellular concentration is higher than the plasma concentration and the rate of exchange between intra- and extracellular pools is very rapid. For most amino acids the turnover time in the intracellular pool is of the order of a 30 minutes to 2 hours.

6) Of all the components of inflow and outflow from the free amino acid pool it appears that the rates of oxidation and transport are the most closely regulated.

1.6.4 Amino Acid Patterns

The plasma amino acid concentration in man and the ratios of free to protein-bound amino acids in rat skeletal muscle are shown in Tables 1.2 and 1.3.

1.6.4.1 The Distribution of Free and Bound Amino Acids

There is no fixed relationship between the pattern of free amino acids in plasma and those found bound within protein (Munro, 1970) and the ratios for the essential amino acids vary over a twenty-fold range. When measuring protein

Table 1.2. Plasma and Intramuscular Water (IMW) Free Amino Acid Concentrations in Man*.

	Plasma (μmol/l)	IMW (μmol/l)	Ratio (IMW/Plasma)
Indispensable			
Histidine	80	370	4.6
Isoleucine	60	110	1.8
Leucine	120	150	1.2
Lysine	180	1150	6.4
Methionine	20	110	5.6
Phenylalanine	50	70	1.4
Threonine	150	1030	6.8
Valine	220	260	1.2
Dispensable			
Alanine	330	2340	7.3
Arginine	80	510	6.5
Asparagine	50	470	9.5
Citrulline	30	40	1.6
Cysteine	110	180	1.6
Glutamate	60	4380	74
Glutamine	570	19450	34
Glycine	210	1330	6.5
Ornithine	60	3000	5.1
Proline	170	830	4.9
Serine	120	980	6.9
Taurine	70	15440	220
Tyrosine	50	100	2.0

*Bergström *et al.*, 1974.

Table 1.3. Bound and Free Amino Acid Concentrations in Rat Muscle*.

	Protein (μmol/g net weight)	Free	Ratio (Protein/Free)
Indispensable			
Histidine	26	0.39	67
Isoleucine	50	0.16	306
Leucine	109	0.20	556
Lysine	58	1.86	31
Methionine	36	0.16	225
Phenylalanine	45	0.07	646
Threonine	60	1.94	31
Valine	83	0.31	272
Dispensable			
Alanine	111	2.77	40
Arginine	67	0.25	269
Aspartate (+amide)	110	1.13	97
Glutamate (+amide)	148	9.91	15
Glycine	117	1.94	60
Serine	74	1.96	38
Tyrosine	36	0.14	226

*Fern, 1975.

synthesis, this difference in composition between the free and bound pools has to be considered: the free amino acid pool cannot be treated as a whole when using a precursor pool to make calculations for protein synthesis. The amino acids used for protein synthesis represent a selection of those in the free pool. During protein synthesis the relative amounts of the amino acids used may vary markedly from their relative distribution in the free pool. The differences in the patterns between the free and the bound pool are maintained by a number of mechanisms. The pattern of amino acids in a balanced diet is broadly similar to that in tissue proteins and hence similar to the amino acid mixture being synthesised or derived from it by its breakdown. In this situation the different pattern that can arise in the free amino acid pool is likely to be due to differences in the relative rates of disposal by oxidation or due to differences in transmembrane transport between amino acids. Because of the values of their Michaelis constants, which are relatively high, the activity of the oxidative enzymes is sensitive to substrate concentration (Krebs, 1972). Therefore, if a substrate has a high steady state concentration, e.g. lysine or threonine, this means that the activity of its oxidative system must be relatively low. The under-representation of the branched chain amino acids in the free amino acid pool may be explained by the high activity of their oxidative enzymes.

1.6.4.2 Diet and the Free Amino Acid Pool

Diets which are deficient in protein can result in a reduction in the plasma concentration of essential amino acids of up to 50% and this is accompanied by

rises of a similar order in the concentration of the non-essential amino acids (Lunn,1976). Starvation however produces changes in the plasma amino acid profile which are opposite to protein deficiency with increases in the concentrations of the essential amino acids and decreases in the concentrations of the non-essential amino acids. The surprising rise in the concentrations of the essential amino acids may be explained by their reduced uptake into protein and increased release from protein breakdown (Millward *et al.*, 1974). In man, the branched-chain amino acids are particularly sensitive to dietary influence, showing the maximal fall amongst all amino acids in response to protein deficiency and the greatest rise in their concentration in muscle in response to fasting.

1.6.4.3 The Dynamic Behaviour of the Free Amino Acid Pool: Turnover Rate

In the rat, the free amino acid pool has been calculated to turn over about once every hour (Munro, 1970). In man, however, the total free essential amino acid pool is smaller than its equivalent in the rat. It also turns over much more slowly (Bergström *et al.*, 1974). There are differences in the turnover rates of individual amino acids in man e.g. the half life of leucine is approximately 45 minutes and that of lysine 10 hours.

1.6.4.4 Uptake of Amino Acids from the Free Amino Acid Pool

In man, the rate of uptake of most amino acids into muscle is proportional to their concentration in plasma (Baños *et al.*, 1973; Lundholm *et al.*, 1987). If cardiac output in adult man is assumed to be 5 l/min and only free amino acids in plasma are taken into account (i.e. ignoring amino acids in red cells) then the total amount of amino acids circulated to the tissues is of the order of 100 mmol/kg/day. This is approximately 8 times the rate at which these amino acids are taken up into protein. In certain situations however there may be a smaller margin of safety. For instance it is possible that amino acid delivery might limit rates of protein synthesis in man with the amino acid having the smallest ratio of delivery to incorporation into protein possibly constraining protein synthesis under conditions of high amino acid demand e.g. during growth spurts or periods of tissue repair after injury, particularly if dietary amino acid intake was concurrently reduced. Taking leucine as an example, the concentration of which in blood is 0.12 mmol/l, if blood flow to total body muscle is approximately 1 l/min and protein synthetic rate is approximately 1.5%/day (Halliday *et al.*, 1988) then the total blood leucine delivery (170 mmol) is about 3.4 times the total amount of leucine which is incorporated into protein daily. It seems possible therefore that under certain conditions of low protein intake, catch-up growth or repair following injury, the supply of amino acids to a tissue might limit the rate of protein synthesis. If similar calculations to that used in the example for leucine are applied to the branched chain amino acids, histidine, methionine and glutamate, it can be calculated that during tissue repair following injury these amino acids may also limit the rate of protein synthesis.

1.6.4.5 Concept of Protein Turnover

The term *turnover* is used to describe the process of renewal or replacement of a given substance. The process itself may involve the production of newly formed material, with the disappearance of some material already present, or it may represent the exchange of material between two or more compartments. Whole body protein turnover is the overall rate at which protein is synthesised or broken down in the body and is the sum of all the individual proteins over the full range from the fastest to the slowest. Generally smaller animals have higher rates of protein turnover per kg body weight and within the same species the young of the species have a higher rate than the old (Young *et al.*, 1975). In the steady state, protein synthesis and breakdown are equal and nitrogen balance is zero.

1.6.4.6 Methods of Assessing Protein Turnover

The main methods used in assessing protein turnover are:

1. *3-Methylhistidine*: a) Assessment of the loss of this amino acid in urine and thereby expression as the ratio of 3-methylhistidine/creatinine, is an index of total myofibrillar protein breakdown in the whole-body (Ballard and Tomas, 1983). b) 3-Methylhistidine release from peripheral tissues (of forearm and leg), when measured and calculated as the product of the arteriovenous difference and blood flow, may be a more specific way to assess skeletal muscle myofibrillar protein breakdown (Rennie and

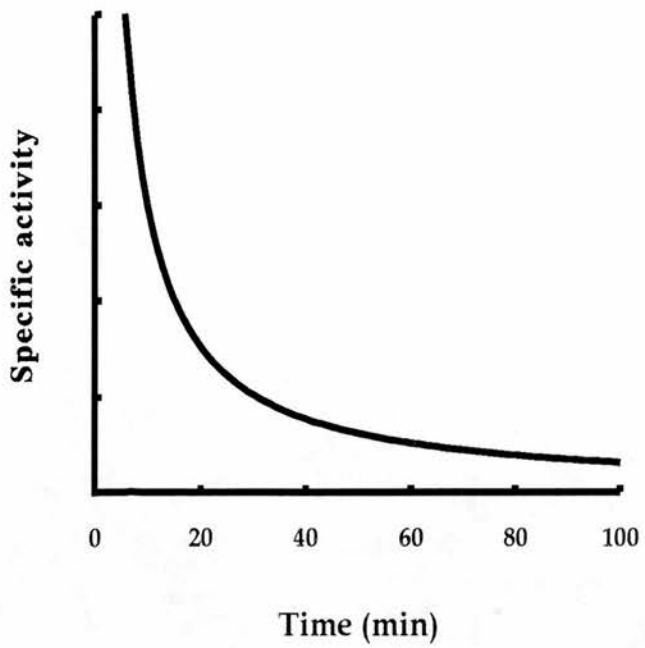

Figure 1.4 Schematic representation of the exponential decay of specific activity over time after administration of a single dose of a radioactive tracer.

Millward, 1983a).

3. Use of a single dose of an isotope labelled amino acid to assess turnover or flux of the amino acid pool from the rate of dilution of the tracer (Figure 1.4); Henriques *et al.*, 1955).

4. Estimation of protein turnover from dilution of [^{15}N] in the end products of metabolism i.e. in urea and ammonia, from labelled non-essential amino acids such as [^{15}N]glycine (Sprinson and Rittenberg, 1949; Olesen *et al.*, 1954).

5. Continuous infusion of a labelled amino acid to assess the flux or turnover

of the amino acid in the free amino acid pool (Figure 1.5). (O'Keefe *et al.*, 1974; Matthews *et al.* 1980; Clarke and Bier, 1982).

5. Techniques using incorporation of labelled free amino acids into the protein-bound amino acid pool within plasma or tissues *in vitro* or *in vivo* (Schoenheimer *et al.*, 1942; Halliday and McKeron, 1975; Rennie *et al.*, 1982a, 1982b).

6. The so-called 'flooding-dose' technique with the use of a single dose of tracer combined with a large dose of non-labelled amino acid to flood the free amino acid pool and thus label the intracellular amino acid pool compartments with tracer to a similar extent as the accessible amino acid in plasma (Garlick *et al.*, 1989).

7. Use of techniques where arteriovenous phenylalanine tracer exchange is measured simultaneously with amino acid balance to assess skeletal muscle protein breakdown and synthesis (Gelfand and Barrett, 1987; Thomson *et al.*, 1989).

8. Measurement of tissue ribosome content to assess *the capacity* for protein synthesis (Millward *et al.*, 1973) or measurement of tissue polyribosomal profile in biopsy samples to assess translational activity (Hammarqvist *et al.*, 1988; Wernerman *et al.*, 1989). Both of these methods are indirect assessments of protein synthesis.

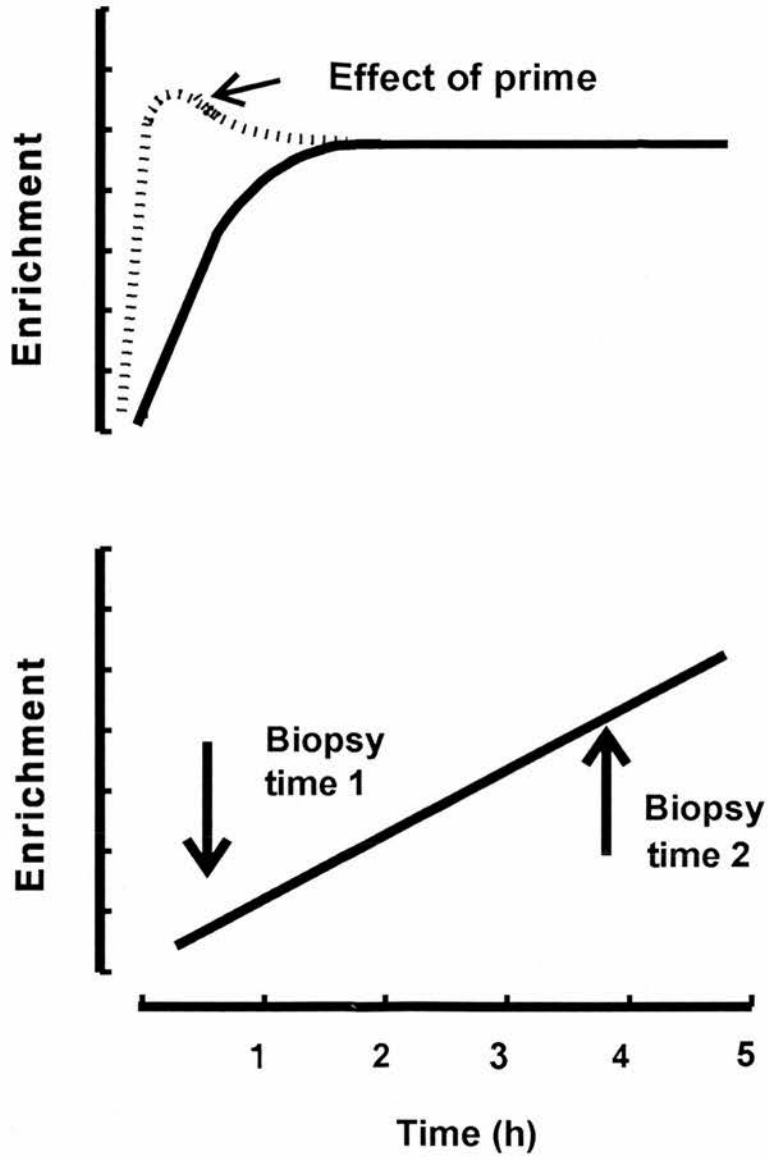

Figure 1.5 The upper panel shows a schematic representation of a continuous infusion of a stable isotope labelled tracer. The lower panel shows the incorporation of the tracer into muscle protein.

1.7 Lean Body Mass: Maintenance, Growth and Wasting

1.7.1 Mechanisms of Growth in Lean Tissues

Growth occurs in lean tissues when the rate of protein synthesis exceeds the rate of protein breakdown. In muscle, growth can be associated with increased turnover where rates of protein synthesis *and* breakdown are both elevated but the increase in the rate of protein synthesis is greater than that of the rate of protein breakdown compared to the basal state (Figure 1.6).

1. During the rapid growth periods of immature animals, the rates of protein breakdown are greater than those of adults of the species (Millward, 1980a; Millward, 1980b). This increase in the rates of protein breakdown in the muscles of immature animals may be related to remodelling of the tissue (Millward, 1975; Maruyama, 1978).
2. An increase in protein breakdown can also occur in muscular hypertrophy e.g. in cardiac muscle related to disease conditions such as systemic hypertension and aortic stenosis, where there are increases in both protein synthesis and breakdown (Gudbjarnason *et al.*, 1964; Zak and Fischman, 1971).
3. During refeeding and recovery from malnourishment, studies in rats have shown that there are increases in protein synthesis and breakdown (Young *et al.*, 1971).

It appears that most forms of rapid growth in skeletal muscle are

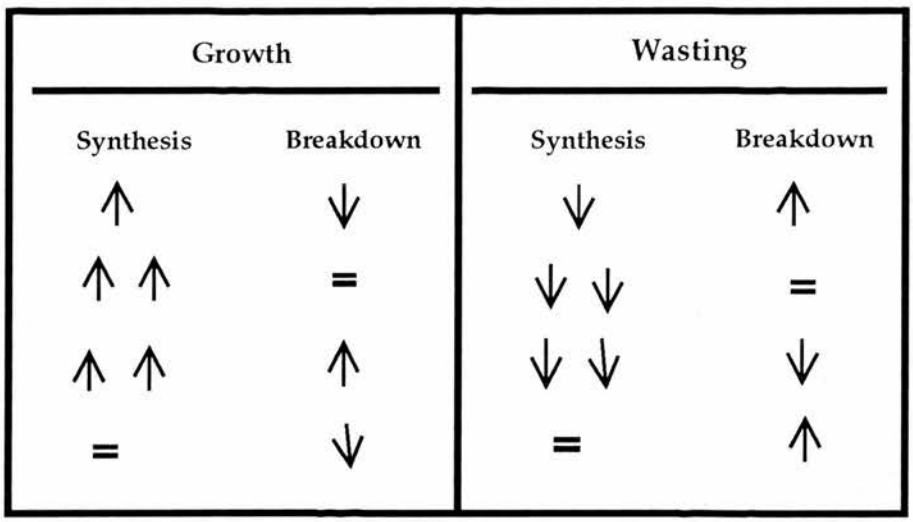

Figure 1.6 Different patterns of muscle protein synthesis and breakdown associated with a variety of conditions affecting muscle growth and wasting.

associated with increases in protein breakdown. Tissue growth can also be associated with a fall in the rate of protein breakdown. This has been demonstrated in a rapidly growing strain of rat (Bates and Millward, 1978).

1.7.2 Mechanisms of Wasting in Lean Tissues

For wasting to occur in lean tissue, an imbalance must exist such that the rate of protein breakdown exceeds the rate of protein synthesis and theoretically

this situation may arise with differing combinations of changes in either process (Figure 1.6). The idea of facilitative and adaptive responses in protein synthesis and breakdown has been proposed by Millward and colleagues to explain the different patterns of turnover (Millward *et al.*, 1976; Rennie *et al.*, 1983b). In skeletal muscle for example, changes in protein mass may occur with alterations in synthesis in an appropriate (i.e. the same) direction but with apparently paradoxical changes in breakdown which would counteract the effect of the change in synthesis. This situation occurs in starvation where the rates both of synthesis and breakdown fall (Millward and Waterlow, 1978) or in hypertrophy induced by work where they both increase (Laurent *et al.*, 1978b). Millward has described these changes as facilitative as they promote the modulation of muscle mass and the changes in breakdown as adaptive as they limit the modulation process. In skeletal and cardiac muscle it is synthesis which is regulated in physiological states and possibly also in pathological states (i.e. facilitative). In other tissues such as liver and gut breakdown appears to be facilitative (Garlick *et al.*, 1973; Emery *et al.*, 1986).

Although there is no full picture of the abnormalities of protein synthesis and breakdown occurring in different pathophysiological situations where muscle wasting occurs in man, there is information available on the patterns of protein turnover in muscle in a number of specific conditions. In patients suffering from cardiac cachexia, for instance, muscle protein breakdown is increased (Morrison *et al.*, 1988a). However, in other conditions involving muscle wasting, muscle protein breakdown is not increased and wasting occurs as a result of a decrease in protein synthesis. This situation arises for example on the first postoperative day in patients who have undergone abdominal surgery (Rennie *et al.*, 1984); in patients

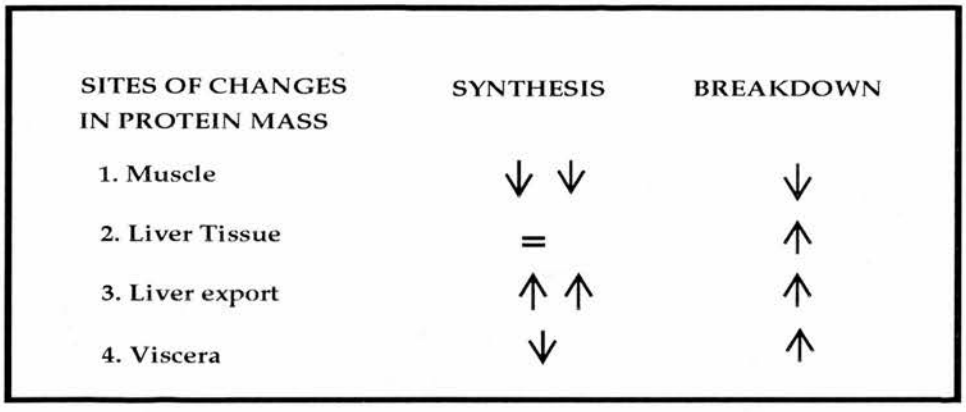

Figure 1.7 Hypothetical contribution of different tissues to whole-body protein turnover (after Rennie, 1985).

with Duchenne muscular dystrophy (Rennie *et al.*, 1982a) and myotonic muscular dystrophy (Halliday *et al.*, 1985); in patients with thyrotoxicosis (Morrison *et al.*, 1988b) and in patients with emphysema (Morrison, *et al.*, 1988a). In cachectic cancer patients muscle wasting is associated with a reduction in protein synthesis and also an accompanying reduction in muscle protein breakdown (Lundholm *et al.*, 1982).

There is only limited information available for man on the contributions of various tissues to whole body protein turnover. Muscle probably contributes around 25-50% of whole body protein turnover in man (Munro, 1970; Rennie *et al.*, 1982b), with the lower figure of 25%, calculated with amended technology (Halliday, 1988) likely to be the better estimate than the earlier suggested figure of 50%. As shown in Figure 1.7, hypothetical simultaneous changes may occur in

different tissues which could be interpreted as an alteration in whole body protein turnover as measured by plasma amino acid flux. It can also be seen that a rise in liver turnover such as that which occurs during stress and following injury may be sufficient to counteract or swamp changes in the opposite direction in muscle when whole body turnover is measured, since liver turnover is more rapid than skeletal muscle (Sakamoto *et al.*, 1983; Emery *et al.*, 1984). The identification of the mechanisms involved in tissue wasting is not purely an academic exercise since it should provide knowledge for future rational therapeutic intervention for chronic wasting conditions (Rennie and Harrison, 1984).

Although physiologically important regulatory processes may adjust homeostatically, there may also be catastrophic unphysiological changes which may be produced by various humoral agents (Wilmore *et al.*, 1974; Rodemann and Goldberg, 1982; Wilmore *et al.*, 1983), neural influences or their lack (Denny-Brown, 1960; Afting *et al.*, 1981), toxins (Powanda, 1977) and cell-mediated factors (Baracos *et al.*, 1983). These factors may be particularly important in sudden acute conditions of wasting rather than chronic. In addition to pathophysiological modulation of the normal mechanisms of protein turnover i.e., synthesis and breakdown, there are two additional processes of considerable importance for the response of lean tissue to disease and injury. These are:

a) Impaired amino acid transport in muscle causing loss of the normal gradient of amino acids between muscle and plasma and loss of the normal concentrative function of amino acid transport in muscle (Wannemacher *et al.*, 1971; Baños *et al.*, 1973; Muhlbacher *et al.*, 1984).

b) Destructive processes resulting in breaching of the cell membrane, influx of

calcium and phagocytic attack (Jackson *et al.*, 1984). These processes are more likely to be important during acute changes due to sepsis and trauma but may also contribute to chronic wasting.

1.7.3 Physiological and Pathophysiological Modulation of Protein Turnover

1.7.3.1 The Effects of Feeding and Fasting

A net uptake of amino acids occurs during feeding and a net release occurs during fasting (Elia and Livesey, 1983). Studies of incorporation of[1-^{13}C]leucine into quadriceps muscle in healthy man show that protein synthesis is greater during feeding than during fasting (Rennie *et al.*, 1982b; Halliday *et al.*, 1988). Similar results have also been obtained by *in vivo* studies in man using exchange of [1-^{13}C,^{15}N]leucine by forearm tissues (Cheng *et al.*, 1985; Cheng *et al.*, 1987). These studies also showed that feeding resulted in an increase in leucine deamination, reamination and oxidation and improved the net balance between synthesis and breakdown but the results suggested that feeding had no effect on the rate of mixed muscle protein breakdown. The lack of any effect of feeding on muscle protein breakdown has also been demonstrated in studies of myofibrillar protein breakdown, as assessed by the limb release of 3-methylhistidine, during parenteral feeding (Tracey *et al.*, 1988). The muscle biopsy and arteriovenous limb-exchange based techniques are disadvantaged in theory by the possibility that changes in the plasma concentration of the amino acid tracee, leucine, which occur

during feeding might alter the tracer equilibrium between the accessible plasma pool and that at the site of incorporation of precursor for protein synthesis within cells. Increases in amino acid transport occurring at increased amino acid concentration (Lundholm *et al.*, 1987) result in increased tracer and tracee exchange between plasma and the intracellular pool (Layman and Wolfe, 1987) and a closer approximation by the plasma enrichment to the intracellular enrichment. Estimates of intracellular amino acid metabolism during postabsorptive conditions, tend therefore to be underestimated when the plasma amino acid enrichment or specific activity is used as a basis for calculations but underestimated to a lesser extent during feeding. It also follows that the tracer dilution technique used in most studies tends to overestimate the increases in protein synthesis during feeding. Where the keto acid of leucine, α-ketoisocaproic acid (αKIC), a measure of intracellular labelling (Matthews *et al.*, 1982; Schwenk *et al.*, 1985a; Layman and Wolfe, 1987; Horber *et al.*, 1989), was used to define precursor pool enrichment in the biopsy-based studies (Rennie *et al.*, 1982b; Halliday *et al.*, 1988), overestimation of the effects of feeding on protein synthesis would occur to a much lesser extent.

Garlick and co-workers, using a flooding dose technique in an attempt to minimise the differences in tracer enrichment between the plasma compartment and the precursor pool for protein synthesis within the cell and thereby reduce the uncertainty about precursor labelling, have reported higher fractional protein synthesis rates in skeletal muscle in man (Garlick *et al.*, 1989) than Halliday and colleagues (Halliday *et al.*, 1988). Garlick and colleagues found no significant effect of feeding on muscle protein synthesis in man using the flooding dose technique (Essén, 1991). It is possible that this discrepancy in the result obtained by the flooding dose technique compared with the stimulation in protein synthesis seen

with feeding when using the primed constant infusion method may be explained by some independent effect, perhaps stimulatory (Buse, 1975; Tischler, 1982; Smith *et al.*, 1992), which the administration of large doses of leucine may have on protein synthesis during these studies and which may mask any acute effects of feeding.

Mixed feeding has been shown to increase whole-body protein synthesis in man by a number of authors (Garlick *et al.*, 1980; Motil *et al.*, 1981a; Motil *et al.*, 1981b; Clugston and Garlick, 1982; Rennie *et al.*, 1982b; Clague *et al.*, 1983; McNurlan *et al.*, 1987), although not by all (Young *et al.*, 1987; Beaufrere *et al.*, 1989). Most of these authors have also reported a reduction in whole-body protein breakdown with feeding (Motil *et al.*, 1981a; Motil *et al.*, 1981b; Clugston and Garlick, 1982; Young *et al.*, 1987; Tracey *et al.*, 1988; Beaufrere *et al.*, 1989) but some authors reported no effect (Garlick *et al.*, 1980 ; Rennie *et al.*, 1982b; Clague *et al.*, 1983; Young *et al.*, 1987). In some of these studies it is possible that significant errors may have occurred because of the recycling of tracer. This problem can lead to an underestimation of protein synthesis where synthesis was measured sequentially following a period of fasting during prolonged tracer infusion (Schwenk *et al.*, 1985b). Failure to take into account metabolism of ingested leucine or protein by gut and liver generally results in overestimates of the reduction in protein breakdown during feeding. Prolonged fasting for a period of four days has been shown to increase whole-body leucine flux or proteolysis (Jensen *et al.*, 1988) and to reduce skeletal muscle protein synthesis in man (Essén *et al.*, 1989). The effect of fasting in the long term is to reduce protein utilization as a source of substrate for gluconeogenesis but such changes take about a week to become apparent (Kettelhut *et al.*, 1988).

1.7.3.2 The Effect of Amino Acids

When mixed amino acids are infused in man, a net increased amino acid uptake has been demonstrated in leg tissues and the splanchnic bed with estimated uptake of the infused amino acid load being 25-30% and 65-70% respectively (Gelfand *et al.*, 1986). No account was taken in this study of release of keto-acids and amino acid metabolites. Infusion of mixed amino acids has been reported to increase whole-body protein synthesis (Castellino *et al.*, 1987; Tessari *et al.*, 1987; Pacy *et al.*, 1988a) although there is no consensus on the effect of mixed amino acid infusion on whole-body protein breakdown. Two studies have demonstrated a decrease in the latter and no change was reported in one study in which use was made of a different kinetic model i.e. a double primary pool as opposed to a reciprocal-pool model (Tessari *et al.*, 1987). Infusion of branched-chain amino acids was shown to have no effect on either net leg protein balance or myofibrillar protein breakdown in healthy subjects and in patients with cardiac cachexia (Morrison *et al.*, 1988a). Branched-chain amino acids also had no effect on protein synthesis assessed by ribosomal profile in man (Hammarqvist *et al.*, 1988; Eriksson *et al.*, 1983), although paradoxically leucine injection is reported to increase the proportion of muscle ribosomes as polyribosomes in fasted rats (Buse *et al.*, 1979). In *in vitro* studies on human muscle, a ten-fold increase in amino acid concentrations alone resulted in an increase in protein synthesis (Lundholm and Schersten, 1977). Also, complete mixtures of amino acids are reported to enhance protein synthesis in cell-free systems (Tyobeka and Manchester, 1985). Leucine is reported to stimulate rat muscle protein synthesis *in vitro* (Buse and Reid, 1975). It is possible that the branched-chain amino acids and particularly leucine are

responsible for the effect of amino acid mixtures on stimulation of muscle protein synthesis and of reductions in muscle protein breakdown (Buse and Reid, 1975; Fulks *et al.*, 1975; Li and Jefferson, 1978; Adibi, 1980; May and Buse, 1989). The mechanism or mechanisms by which such effects are mediated are not known as the k_m for the charging of *t*RNA is likely to be much lower than the intracellular concentration of amino acids so that the *t*RNA is always fully charged (Shenoy and Roger, 1978; Flaim *et al.*, 1982a, 1982b; Tyobeka and Manchester, 1985). The observation of a reduction in ribosomal subunits during provision of branched-chain amino acids (Li and Jefferson, 1978; Buse *et al.*, 1979) appears to indicate an effect of amino acids on the initiation of protein synthesis. In addition to an increase in muscle protein synthesis, *in vitro* studies in rats demonstrate that mixtures of amino acids reduce muscle protein breakdown (Fulks *et al.*, 1975; Frayn and Maycock, 1979).

1.7.3.3 Effects of Substrates

There is little information on the effects of different substrates on protein turnover in man. *Glucose* infusion at a rate of 4 mg/kg/min is reported to reduce whole-body protein breakdown in healthy man with no effect on protein synthesis (Robert *et al.*, 1984). In addition increases in plasma glucose and insulin concentrations, there was a trend toward a fall in glucagon concentrations, so that it is possible the effects of glucose provision were mediated indirectly by such alterations in hormone levels. In another study, feeding large doses of energy nutrients of either carbohydrate, fat or a mixture of both improved nitrogen balance

and decreased leucine oxidation (Motil *et al.*, 1981b). These induced a reduction in protein breakdown but no alteration in protein synthesis. Experiments with rat muscle *in vitro* have shown that low concentrations of glucose in the perfusing media results in increased leucine oxidation compared to a period of glucose replacement (Buse *et al.*, 1972) and glucose alone has been reported to increase muscle protein synthesis (Hedden and Buse, 1982).

The ketone body β-*hydroxybutyrate* has been suggested to increase muscle and whole-body protein synthesis and to reduce whole-body leucine oxidation in man (Nair *et al.*, 1988b). It is possible that these effects in fact resulted from changes in acid-base homeostasis, a factor shown to have effects on protein turnover and amino acid oxidation in dogs (Rodriguez *et al.*, 1989). There is no apparent effect of β-hydroxybutyrate on whole-body protein breakdown (Miles *et al.*, 1983; Nair *et al.*, 1988b).

Infusion of medium-chain *triglycerides* and long-chain triglycerides in man improves the net amino acid balance across forearm suggesting either increased protein synthesis or reduced proteolysis of skeletal muscle (Wicklmayr *et al.*, 1987). Increases in plasma fatty acid concentrations in dogs reduced whole-body leucine flux and leucine oxidation and tended to reduce whole-body protein synthesis (Tessari *et al.*, 1986). Conversely, decreased plasma free fatty acid concentrations increased whole-body leucine flux and oxidation and decreased whole-body protein synthesis (Tessari *et al.*, 1986).

1.7.3.4 The Effect of Hormones

Information on the effect of hormones on protein turnover is relatively sparse. The effects of *insulin* on protein turnover have been studied for some time. Insulin acts on a membrane receptor which possesses intrinsic tyrosine kinase activity (Kahn and White, 1988). The events within the cell which occur after insulin binds are not clearly established but appear to result from phosphorylation of intermediates. In young animals, provision of insulin stimulated muscle protein synthesis (Jefferson, 1980; Kimball and Jefferson, 1988). This stimulation could only be observed *in vitro* when substantial concentrations of amino acids were made available in the incubation medium. In sheep, insulin alone did not stimulate muscle protein synthesis (Oddy *et al.*, 1987. Muscle protein breakdown has been shown to be sensitive to insulin (Gelfand and Barrett, 1987; Bennet *et al.*, 1988; Arfvidsson *et al.*, 1989) although recent evidence appears to suggest this finding may be artefactual (Biolo *et al.*, 1995). It has been suggested that the provision of insulin alone effectively empties the pool of free amino acids in skeletal muscle as documented in normal subjects and in patients with uraemia and diabetes (Alvestrand *et al.* 1988; Bennet *et al.*, 1990b) and the provision of small amounts of amino acids is insufficient to provide enough substrate during insulin infusion; only when amino acid concentrations are elevated to the extent seen after a large meal will limbs of normal and diabetic subjects achieve net positive amino acid balance by a process of inhibition of protein breakdown and stimulation of protein synthesis (Bennet *et al.*, 1990a; Bennet *et al.*, 1990b). However, Biolo and co-workers (1995), by simultaneous use of biopsy and arteriovenous exchange techniques, have clearly demonstrated a stimulatory effect of insulin on skeletal

muscle protein synthesis, with no effect on breakdown, in healthy post-absorptive subjects.

Growth hormone has substantial influences on increases in lean body mass during growth (Tanner *et al.*, 1977) especially at puberty and excretion of 3-methylhistidine in patients with pituitary lesions appears depressed. It also improves post-operative nitrogen balance in man (Manson and Wilmore, 1986; Ponting *et al.*, 1988). In pharmacological doses in healthy man, growth hormone decreases whole-body leucine oxidation and increases whole-body protein synthesis but appears to have no effect on whole-body protein breakdown (Horber and Haymond, 1989). In animal studies growth hormone appears to exert its overall anabolic action on muscle protein turnover by increasing muscle protein synthesis (Cameron *et al.*, 1988).

The effects of growth hormone are predominantly mediated by the two *insulin-like growth factors I and II* (IGF-I and IGF-II) (Salmon and Daughaday, 1957). It is also likely that the anabolic effect of growth hormone is mediated by insulin-like growth factor I (Isgaard *et al.*, 1989) and this factor is a potent stimulator of protein synthesis *in vitro* (Froesch *et al.*, 1985). In contrast, intravenous infusion of IGF-I in rats appeared to depress whole-body protein synthesis and breakdown and reduced the rates of protein synthesis in a number of specific tissues (Jacob, 1989). These effects may have arisen from use of supraphysiological doses, where the IGF-I was not associated with its specific binding protein and a direct effect mediated via the insulin receptor may have resulted. It has been shown that growth hormone has greater growth promoting effects than IGF-I suggesting that additional factors other than IGF-I are important in mediating the effects of growth hormone (Skottner *et al.*, 1987).

The glucocorticoids and *cortisol* in particular, have a negative effect on protein balance. In man short courses of cortisol increase whole-body protein breakdown (Beaufrere *et al.*, 1989) and stimulate transfer of nitrogen from leucine to alanine and to glutamine with a resultant increased synthesis of these gluconeogenic amino acids (Simmons *et al.*, 1984; Darmaun *et al.*, 1988). In patients however with hyperglucocorticoidaemia of longer duration from untreated Cushing's syndrome, an increased whole-body protein breakdown was not observed (Tessari *et al.*, 1989). It is possible that these different effects of cortisol in the longer term are the result of adaptive mechanisms such as increased insulin concentration and to a predominant action of glucocorticoids in impairing protein synthesis. Animal studies show that corticosteroids may increase both myofibrillar protein breakdown and decrease protein synthesis (Rannels and Jefferson, 1989; Odedra *et al.*, 1983; Kayali *et al.*, 1987) but a study in man has shown no effect on myofibrillar protein breakdown (Beaufrere *et al.*, 1989). Glucocorticoids decrease glutamine concentrations in plasma and muscle (Muhlbacher *et al.*, 1984). This may be the indirect mechanism by which they influence protein turnover as glutamine itself has a role in regulating protein turnover in injury (Rennie *et al.*, 1986). In healthy man, infusion of adrenaline decreases muscle ribosome concentration (Wernerman *et al.*, 1989) and decreases whole-body protein breakdown, leucine oxidation and stimulates *de novo* alanine synthesis (Miles *et al.*, 1984; Kraenzlin *et al.*, 1989). These may be direct effects or possibly indirect effects mediated by alterations in insulin concentration. Muscle protein breakdown appears to be reduced by adrenaline (Kraenzlin *et al.*, 1989).

Excessive *thyroxine* levels in man results in a depression of skeletal muscle protein synthesis but an unaltered myofibrillar protein breakdown, as assessed by

the increased arteriovenous net efflux of tyrosine and unaltered 3-methylhistidine balance across the leg (Morrison *et al.*, 1988b). Although Morrison and colleagues did not demonstrate any significant effect of hypothyroidism on leg protein balance, in animal studies this condition results in reduced protein synthesis by muscle (Brown *et al.*, 1981), corrected by treatment with triiodothyronine (Brown and Millward, 1983).

In the insulin deficient state *glucagon* increases whole-body protein breakdown and leucine oxidation in man (Nair *et al.*, 1987). Although it has been suggested that glucagon inhibits muscle protein synthesis *in vivo*, such an effect is only seen at pharmacological doses (Preedy and Garlick, 1988). There is no effect of glucagon on muscle ribosome concentration or on the proportion of ribosomes found as polyribosomes in man (Wernerman *et al.*, 1989).

In patients with myotonic dystrophy who are hypogonadal, *testosterone* administration appears to increase muscle protein synthesis (Griggs *et al.*, 1986). However, although the same workers reported significant increases in lean body mass and protein synthesis estimated from incorporation of [^{13}C]leucine into muscle protein sampled by muscle biopsy (Griggs *et al.*, 1989), examination of the individual values suggests that the response is very variable and only in one or two subjects did large biological effects occur.

Tumour necrosis factor-α , a polypeptide hormone produced by lymphocytes and macrophages, appears to increase skeletal muscle protein breakdown in cancer patients, as assessed by net amino acid efflux from the limb, yet conversely increases whole-body protein synthesis (Warren *et al.*, 1987; Starnes *et al.*, 1989). *In vitro* studies have generally failed to confirm such an effect of increased muscle protein breakdown (Evans *et al.*, 1989), suggesting that the effects of tumour

necrosis factor-α may be mediated indirectly, e.g by increases in cortisol or glucagon. There is generally thought to be no effect of interleukin-1 on protein turnover but a related factor, interleukin-6, may have a stimulatory effect on muscle protein breakdown (Evans *et al.*, 1989).

1.7.3.5 Gender-related Differences in Protein Turnover

There are insufficient data available in the literature to determine whether or not there are significant gender related differences in muscle protein synthesis but 3-methylhistidine excretion studies do show a slight difference with the rate of turnover being less in women than in men (Young and Munro, 1978). It is difficult to interpret the fractional rates of protein turnover in relation to creatinine because of sex hormone-influenced differences in creatinine metabolism, which will inevitably affect the 3-methylhistidine/creatinine urinary ratio.

1.7.3.6 The Influence of Exercise and Immobility on Protein Turnover

It is well known that muscular contractile activity or the lack of it can induce changes in muscle composition and muscle mass (Tucker *et al.*, 1973; Laurent *et al.*, 1978a, 1978b; Watson *et al.*, 1984). The relatively sparse information available for man regarding the related changes in protein turnover suggests that muscle protein synthesis is depressed during immobilisation (Gibson *et al.*, 1987) and that mild electrical stimulation at levels sufficient to produce only

5% of maximum voluntary contraction for 1 h a day is sufficient to halt the loss of muscle (Gibson *et al.*, 1988). Electrical stimulation of muscle in paraplegic subjects also increased the rate of muscle protein synthesis in 5 subjects out of 6 (Pacy *et al.*, 1988b).

1.7.3.7 General Anaesthesia

To enable accurate interpretation of results from studies in protein turnover involving anaesthesia and surgery it is important to initially clarify the separate effect of anaesthesia. Intravenous anaesthetics themselves initiate negligible endocrine-metabolic changes compared to the effects of surgical trauma (Nistrup *et al.*, 1976; Oyama, 1980; Tarhan *et al.*, 1971). The duration of anaesthesia itself does not appear to contribute to the response of interleukin-6 observed in surgical patients (Cruickshank *et al.*, 1990). Thiopental combined with a low dose of fentanyl blunts the stress response to endotracheal intubation (Dahlgren *et al.*, 1981; Giesecke *et al.*, 1988). In itself, mechanical ventilation is known to initiate an endocrine response, including a release of antidiuretic hormone in intensive care patients (Hemmer *et al.*, 1980). After the induction of neuroleptic anaesthesia the exchange of free amino acids across a leg is unaltered (Stjernström *et al.*, 1986). Whole body protein synthesis and degradation are decreased by halothane, when determined before surgery (Rennie and Maclennan, 1985). In contrast, enflurane administration during the 2 hours before surgery does not affect whole body protein synthesis or breakdown (Carli *et al.*, 1989). The effects of different anaesthetic agents on protein synthesis in human individual tissues have so far not been

investigated. In animal experiments, halothane is the most frequently studied anaesthetic agent, since it is easy to quantify and to use. In intubated, mechanically ventilated dogs whole body protein synthesis was decreased by halothane anaesthesia administered with nitrous oxide and oxygen (Horber *et al.*, 1988). In spontaneously breathing rats protein synthesis decreases in liver and lung when halothane is given in 66% nitrous oxide or in O_2-enriched air (f_iO_2=0.33). Only protein synthesis in the lung is affected by fentanyl/midazolam with 21% oxygen. Protein synthesis in skeletal and cardiac muscle is unaffected by halothane and fentanyl/midazolam (Heys *et al.*, 1989). Also, albumin synthesis decreases when halothane in 40% oxygen is given to rats twice within 24 hours, but is unaffected after a single session of halothane anaesthesia (Franks *et al.*, 1988). Thus, these studies in rats appear to indicate that the effect of anaesthesia is dependant upon both the agent used and the tissue studied.

1.7.3.8 Surgical injury

Even though it has been accepted for a long time that surgical trauma initiates loss of muscle protein (Cuthbertson, 1930), the catabolic effects have mostly been characterised one or several days postoperatively. Metabolically, the immediate perioperative period is recognised to be essential, since surgery rapidly evokes an endocrine response with increased levels of stress hormones and an augmentation of other active factors such as cytokines, which all might act as regulators for protein catabolism (Cruickshank *et al.*, 1990; Giesecke *et al.*, 1988; Rutberg *et al.*, 1984). During surgery, an increase in the release of free amino

acids from the leg is observed, indicating net muscle protein catabolism (Stjernström *et al.*, 1986). Muscle protein synthesis decreases at 24 hours postoperatively, as reflected by the configuration and size distribution of ribosomes (Wernerman *et al.*, 1986). At the same time the efflux of 3-methylhistidine from the leg decreases markedly, indicating a fall in muscle protein degradation, and the tyrosine efflux doubles, suggesting an even larger decrease in protein synthesis as compared to degradation (Rennie *et al.*, 1984). Also, the free amino acid concentrations in muscle show a characteristic catabolic pattern during the first post operative day (Essén *et al.*, 1991), that further develops during the ensuing two to three days (Vinnars *et al.*, 1975). On the third postoperative day protein synthesis is still decreased when reflected by the ribosome technique (Wernerman *et al.*, 1986). Several isotopic studies have elucidated the effect of surgery on whole body protein metabolism. Immediately after surgery of moderate severity whole body protein synthesis and degradation decrease (Carli *et al.* 1989). One to several days after surgery of intermediate nature whole body protein synthesis decreases, while protein degradation is either unaffected (Crane *et al.*, 1977; Kien *et al.*, 1978; O'Keefe *et al.*, 1974) or increased (Clague *et al.*, 1983). Whole body protein synthesis is shown to increase after surgery of moderate severity (Harrison *et al.*, 1989; Lowry *et al.*, 1986) and after major trauma (Birkham *et al.*, 1980; Birkham *et al.*, 1981). In major trauma, however, protein degradation increases even more than protein synthesis (Birkhahn *et al.*, 1980; Birkhahn, *et al.* 1981).

1.7.4 The Effect of Intravenous Parenteral Nutrition

1.7.4.1 Parenteral Nutrition: a historical note

The concept of parenteral nutrition is not a new one. Milk was given intravenously to combat the wasting associated with cholera by Hodder in Toronto in 1873 (Johnston, 1978). In 1913 Henriques and Anderson (Johnston, 1978) carried out the first successful experimental studies using goat muscle digested with pancreatic extract and combined with glucose, sodium and potassium. They were able to maintain a positive nitrogen balance in dogs for 6 days using this mixture. The first step in clinical intravenous nutrition was the use of sterile solutions of 5% dextrose during the First World War. By the late 1930's protein hydrolysates were under extensive study by Elman (Johnston, 1978) and it was not long until the first fat emulsions were being prepared for intravenous use. The next major advance in intravenous nutrition was the demonstration by Dudrick in 1967 that the simultaneous administration to puppies of glucose and amino acids with added vitamins and minerals as the sole source of nourishment allowed normal muscular and skeletal growth to occur. The subsequent development of total parenteral nutrition (TPN) as a practical clinical proposition came when safe techniques were devised for the administration of hypertonic nutrient solutions into the superior vena cava (Dudrick *et al.*, 1968). The clinical use of TPN has led to significant advances in surgical practice with a growing appreciation of the problems of malnutrition in surgical patients, their adverse effect on post-operative recovery and how they may be successfully managed (Wilmore and Dudrick, 1968).

1.7.4.2 Postoperative Nutrition

The postoperative period is often characterised by semistarvation due to anorexia or to restricted nutritional intake for medical reasons. Various types of artificial nutrition may be used to overcome this semistarvation and TPN is recommended when the intestinal tract cannot be utilized for longer periods. However, the efficacy of TPN in the post-traumatic period is not fully evaluated in terms of clinical outcome or biochemical effects. Despite TPN, a negative nitrogen balance is observed in normal nourished subjects after surgery of moderate severity (Hammarqvist *et al.*, 1989; Hammarqvist *et al.*, 1991; Wernerman *et al.*, 1987). After major trauma the release of amino acids across the forearm as well as the urinary excretion of 3-methylhistidine increases, in spite of postoperative TPN (Finley *et al.*, 1986). The effects of surgical trauma on the muscle free intracellular amino acids and on the concentration and size distribution of ribosomes in muscle are not reversed by postoperative TPN (Vinnars *et al.*, 1980; Wernerman *et al.*, 1986). However, conventional TPN might be inadequate for the needs of the traumatised patient. Studies on the effect of postoperative TPN on whole body protein synthesis have given discordant results, showing either an increase (Clague *et al.*, 1983; Lowry *et al.*, 1986; O'Keefe *et al.*, 1981) or a decrease (Crane *et al.*, 1977; Kien *et al.*, 1978). This may be due to differences in i) the severity of the trauma ii) the nutritional status of the patients iii) the postoperative nutritional regimens and iv) the methods used for the investigations.

Although initially the amino acid solutions used in TPN were made from hydrolysed protein, reflecting the protein concentration, present-day practice involves the use of crystalline amino acids made to a specific formulation which

includes all the indispensable amino acids in appropriate amounts together with a variety of dispensable amino acids. The so-called dispensable amino acids do, in fact, appear to play a useful role in amino acid metabolism since growth in animals deprived of them is less in their absence. Also, recently it has become obvious that glutamine is one dispensable amino acid which may be less dispensable than previously thought.

1.8.1 The Intracellular Glutamine Pool: Its Role in Health and Disease

In the early 1970's reliable data on intracellular concentrations of free amino acids in human muscle tissue first became available (Bergström *et al.*, 1974). Of the total pool of free amino acids glutamine was found to constitute 61%, and its intracellular concentration was in the order of 19.5 mmol/l intracellular water. The intracellular/extracellular transmembrane gradient of 34 indicated that the diffusion of glutamine through the muscle fibre membrane is restricted. The results of this early study confirmed previous findings in experimental animals showing that the intracellular glutamine concentration is much higher than that in the extracellular compartment (Herbert *et al.*, 1966; Munro 1970). The data, however, also emphasized the consistent differences in amino acid patterns found between species.

Skeletal muscle is the main tissue site for glutamine synthesis and glutamine is released continuously from it (Karl *et al.*, 1976; Marliss *et al.*, 1971). The efflux of amino acids from muscle tissue increases following trauma. Glutamine and alanine represent the major portion of the total amino acid release

during stress (Aulick and Wilmore 1979; Clowes *et al.*, 1980; Lund 1985). The mechanisms underlying the diminished intracellular concentration of free glutamine in muscle are not entirely understood. Accompanying trauma the activity of glutamine synthetase in rat skeletal muscle increases, promoting an elevated production of glutamine (Ardawi, 1988). An unchanged activity of glutamine synthetase is reported in dogs during administration of corticosteroids, that parallels an increased glutamine outflow from muscle and decreased glutamine levels in skeletal muscle tissue (Mühlbacher *et al.*, 1984). An increased outflow of glutamine from skeletal muscle, which exceeds the rate of glutamine synthesis, is a plausible explanation for the decreased concentration in muscle tissue. The non-hepatic splanchnic organs, particularly the small intestine, show an increased uptake of glutamine accompanying trauma (Clowes *et al.*, 1980; Felig 1973). Glutamine constitutes a carrier for amino acid and ammonium nitrogen as well as carbon skeletons emanating from amino acid deamination in skeletal muscle. A sodium dependent carrier mechanism for glutamine across the cell membrane has been proposed (Hundal *et al.*, 1987; Rennie *et al.*, 1986). It has been suggested that this transport mechanism is influenced by the hormonal environment and by concentrations of electrolytes, branched chain amino acids and glutamine. The intracellular influx of sodium may thus be linked with the efflux of glutamine and lead to a decline in the intracellular glutamine concentration. This hypothetical association is supported by observations of an increased concentration of sodium in muscle after major trauma (Larsson *et al.*, 1984) and also after cholecystectomy (Stillström *et al.*, 1987).

A fall in the intramuscular free glutamine concentration accompanies a number of catabolic conditions including trauma, sepsis and elective surgery

(Askanazi *et al.*, 1978; Askanazi *et al.*, 1980a; Milewsky *et al.*, 1982; Essén *et al.*, 1988; Legaspi *et al.*, 1988; Roth *et al.*, 1982; Roth *et al.*, 1985; Roth *et al.*, 1987; Vinnars *et al.*, 1975; Vinnars *et al.*, 1976; Wernerman *et al.*, 1987). Following surgical injury this fall is apparent on the first postoperative day (Essén *et al.*, 1988) and in many cases by the third or fourth postoperative day it is responsible for a depression of up to 60% in the intramuscular free glutamine concentration (Fürst, 1984). It is of interest that while provision of large amounts of amino acids normalized the non-essential amino acid pool, the intracellular free glutamine level remained low (Fürst *et al.*, 1979). Thus, in patients receiving conventional TPN with amino acids, the decrease in glutamine was accompanied by an accumulation of the other non-essential amino acids. The uniform reduction of approximately 50-60% in the muscle free glutamine pool is one of the most typical features of the response to catabolism. The extent of the intracellular glutamine depletion seems to be virtually obligatory, irrespective of the magnitude or severity of catabolism. Depletion of this major intracellular non-essential nitrogen source might be a limiting factor for optimum protein synthesis (Fürst *et al.*, 1987). Manipulations of the free glutamine concentration in animal experiments lead to changes in protein synthesis in skeletal muscle tissue. *In vivo* studies in rats show that there is a good correlation between the rate of muscle protein synthesis and the intramuscular free glutamine concentration (Jepson *et al.*, 1988). Evidence to suggest that this effect is a causal one has been provided by demonstration that manipulation of intramuscular free glutamine concentration in the perfused rat hind limb causes an increase in muscle protein synthesis (Maclennan *et al.*, 1987). In addition, muscle protein breakdown, as assessed by [^{15}N]phenylalanine dilution, may be depressed by increased glutamine availability although myofibrillar protein

breakdown appears not to be, according to the extent of 3-methylhistidine release (MacLennan *et al.*, 1988). In dogs, following trauma, provision of large amounts of amino acids with or without glutamine preserves the muscle free glutamine concentration and improves nitrogen balance across skeletal muscle (Kapadia *et al.*, 1985). However, a supply of glutamine together with elevated insulin levels in non-traumatized animals does not influence the *in vivo* protein synthetic rate in muscle (Garlick and Grant, 1988). A combined infusion of stress hormones simultaneously decreases the concentrations of polyribosomes and free glutamine in skeletal muscle in healthy volunteers (Wernerman *et al.*, 1989; Wernerman *et al.*, 1987). Furthermore, administration of corticosteroids to animals increases the efflux of glutamine from skeletal muscle (Mühlbacher *et al.*, 1984) and stimulates the uptake of glutamine from blood across the intestine (Souba *et al.*, 1985a, 1985b).

An increased uptake of glutamine by the small intestine has also been shown to occur intraoperatively (Felig *et al.*, 1973; Lund, 1985). The small intestinal mucosa is dependant on a continuous supply of glutamine as respiratory fuel and for normal cell division (Souba *et al.*, 1985; Windmueller and Spaeth, 1974). Of the glutamine consumed by the intestine in rats in normal conditions a large part is metabolised in the intestinal lumen (Windmueller, 1975). If the availability of glutamine is low, the mucosal barrier may become impaired (Baskerville *et al.*, 1980), and allow the entrance of endotoxins and bacteria (Wilmore *et al.*, 1988). In trauma, the increased extraction of glutamine from blood by the splanchnic organs is paralleled by an increased glutamine efflux from muscle tissue (Felig, 1975; Lund, 1985). This may constitute a pathophysiological response in trauma in order to maintain normal intestinal epithelial structure to serve as a barrier against bacterial migration (Wilmore *et al.*, 1988). Animal

experiments show that the administration of enteral nutrition prior to stress inhibits the early corticosteroid response to bleeding (Ljungqvist *et al.*, 1986). It is possible therefore that following trauma, the intestine may be better supported by adding glutamine or its analogues to conventional TPN.

1.8.2 The Effect of Nutrition on the Free Intracellular Glutamine Pool

In several studies, intramuscular free amino acids have been measured following elective operations (Vinnars *et al.*, 1975 and 1976; Askanazi *et al.*, 1980b; Vinnars *et al.*, 1983) and after severe accidental injury and burns (Vinnars *et al.*, 1976; Fürst *et al.*, 1979). The effects of different parenteral nutrition regimens with various amounts and proportions of amino acids were evaluated in the immediate post-injury phase (Vinnars *et al.*, 1983). The pattern of changes in intracellular free amino acids after trauma (Askanazi *et al.*, 1980a and 1980b) was not significantly influenced by parenteral nutrition. Alterations in the individual amino acid concentrations appeared not to be affected by the total amounts or proportions of amino acids administered. Surprisingly, the high amounts of amino acids given were not reflected in an increased free amino acid pool in muscle and, importantly, the substantial decrease in muscle glutamine concentrations was found in all patients in spite of such amino acid provision. The reason why the intracellular glutamine pool is depleted in the catabolic state remains obscure. Increased glutamine transport, due to accelerated gluconeogenesis in the liver may serve as a possible explanation. Also, hormonal factors may have an important role: in one study, intracellular amino acid measurements indicated that glucagon-

induced proteolysis in the liver is mediated by intracellular depletion of glutamine (Schworer *et al.*, 1979). Under normal conditions both liver and muscle release glutamine while uptake by the kidney and gastrointestinal tract quantitatively balances this release (Windmueller *et al.*, 1974; Cahill *et al.*, 1975; Windmueller 1974). More recent evidence suggests that in a post-injury state the liver switches from glutamine release to glutamine balance or slight uptake (Souba *et al.*, 1983). The increased amount of glutamine released in a catabolic state is taken up by the gut while kidney exchange remains unaltered. The gastrointestinal tract may therefore be considered as an important regulator of amino acid flux following injury (Souba *et al.*, 1983; Souba *et al.*, 1985) and glutamine might be contemplated as an important and appropriate substrate not only for the skeletal muscle pool but also for the gastrointestinal tract (Souba *et al.*, 1985; Cersosimo *et al.*, 1986).

1.8.3 Glutamine Supplementation of TPN

There appears to be sufficient evidence to suggest that glutamine supplementation of parenteral solutions might be beneficial in the catabolic state. However there has been concern regarding the inclusion of free glutamine in such solutions because of its instability during storage and heat-sterilization (Meister, 1956; Stehle *et al.*, 1984; Stehle *et al.*, 1987). The possibility of substituting currently available amino acid solutions with suitable di- and tripeptides has recently become feasible (Fürst, 1987). This has provided an opportunity for supplying glutamine intravenously by means of infusing stable and soluble glutamine-containing peptides.

1.9.1 L-alanyl-L-glutamine Dipeptide Synthesis and Characterization

A dipeptide of glutamine, L-alanyl-L-glutamine (Ala-Gln) has been synthesised by applying the N-carboxy anhydride method in the aqueous phase (Stehle *et al.*, 1982). The purity in the final product approached 100% and its structure could be fully confirmed by field-desorption mass spectrometry and proton magnetic resonance spectrometry (Stehle *et al.*, 1982a; Stehle *et al.* 1982b). The synthetic peptide Ala-Gln is highly soluble (568 g/l H_2O; 20°C) and stable during heat sterilization over a wide pH range. The solubility of Ala-Gln is 16-fold higher than that known for the free amino acid glutamine (36 g/l H_2O; 20°C). The stability of Ala-Gln during storage and heat-sterilization has been investigated in some depth, particularly in view of the reported instability of free glutamine (Meister, 1956; Stehle *et al.*,1984; Stehle *et al.*, 1987). When Ala-Gln was heat-sterilized for 30 and 60 min at 120°C at various pH no liberation of ammonia or formation of pyroglutamic acid or L-alanyl-L-glutamic acid was observed (Stehle *et al.* 1984; Fürst *et al*, 1987, Stehle *et al.*, 1985). Thus, the synthetic dipeptide Ala-Gln appears to be a stable compound suitable for the heat-sterilisation process which would be required prior to considering its inclusion in future parenteral solutions.

1.9.2 *In Vivo* Utilization of L-Alanyl-L-Glutamine: Animal Studies

Isotope studies with (Ala-[U^{14}C]Gln) administration in the rat and dog strongly indicate that the dipeptide is easily available and the constituent amino acids are rapidly used for protein synthesis, preferentially in muscle tissue. In catabolic rats, continuous TPN without inclusion of Ala-Gln resulted in a profound decrease in tissue free glutamine levels compared with normal rats (Fürst *et al.*, 1987). Inclusion of Ala-Gln in TPN was followed by an increase in the tissue free glutamine pool, considerably in liver and markedly in muscle (Fürst *et al.*, 1987). These findings indicate a preferential capacity of muscle tissue for uptake of Ala-Gln and suggest subsequent utilization of the liberated free glutamine in this tissue.

Synthesis of ^{14}C-labelled Ala-Gln (Ala-[U^{14}C]Gln) facilitated studies of *in vivo* cleavage and subsequent utilization of the dipeptide in rats (Amberger *et al.*, 1983a; Amberger *et al.*, 1983b; Amberger *et al.*, 1984; Amberger *et al.*, 1985). After a bolus injection of Ala-Gln (Ala-[U^{14}C]Gln) the course of labelling was followed in non-protein and protein fractions of plasma, liver, kidney and muscle specimens as well as in the urine (Amberger *et al.*, 1983a, 1983b). In a separate study the rate of oxidation was determined by means of $^{14}CO_2$ expiration (Amberger *et al.*, 1984). The release of $^{14}CO_2$ peaked at 55 min after injection and declined rapidly during the next two hours. The recovered radioactivity after three hours calculated as a percent of injected dose was 49%. This result provided the first evidence for *in vivo* utilization of the peptide Ala-Gln, suggesting that about 49% of released Gln was available as an energy substrate.

In the liver and kidney non-protein fractions, the high initial labelling was

followed by a subsequent rapid decline. In contrast, the initially moderate labelling of the muscle non-protein fraction showed a progressive increase, suggesting a continuous active transport of the label to this fraction. This enrichment of the muscle free pool may have occurred as a result of redistribution of radioactivity presumably arising from the plasma non-protein fraction or from other tissue pools.

The rapid decline of label in the liver and kidney non-protein fractions was accompanied by an increase in the measured radioactivity in the corresponding protein fractions (Amberger *et al.* 1983a, 1983b; Fürst *et al.*, 1987). Liver protein displayed an initial high labelling, indicating a rapid uptake and subsequent metabolism of the glutamine dipeptide in this tissue. Interestingly, the enrichment of liver protein attained its maximum with a simultaneous rapid increase of the label in plasma protein fractions. This observation may suggest liver-specific synthesis of plasma proteins. A significant and steadily increasing incorporation was apparent in the muscle protein fraction. This observation suggested not only a high capacity of muscle tissue to take up the glutamine peptide but also indicated an efficient subsequent intracellular utilization of the liberated free glutamine in this tissue.

The relative distribution of the recovered label in the rat three hours after injection is depicted in Table 1.4. $^{14}CO_2$ accounted for the main part of the recovered label (49%). After this, however, muscle revealed the major portion of the recovered radioactivity (33%) in comparison with other investigated tissues (liver: 9%, plasma: 5.5% and kidney: 1%). Urinary loss of the label was lower than 3%.

These results are in good agreement with those obtained in dogs (Amberger *et al.*, 1985; Fürst *et al.*, 1987) and emphasise the predominant quantitative role

of muscle in utilizing the dipeptide Ala-Gln. Indeed, the high percentage of radioactivity found in muscle tissue is likely to reflect the greater proportion of muscle mass compared with other organ tissues.

Since previous efforts to normalize the post-operative intra-cellular glutamine pool by using alternative non-essential nitrogen sources have failed (Vesterberg *et al.,* 1985), the obvious question which arises is whether or not a maintenance of the intracellular glutamine pool is feasible following parenteral provision of glutamine or glutamine-containing peptides. Wilmore and his colleagues (Kapadia *et al.,* 1985; Johnson *et al.*, 1986) infused amino acid solutions with or without glutamine enrichment to dogs during standard surgical trauma or during simulated stress. By replacing ample amounts of nitrogen as glutamine, they were able to maintain muscle free intracellular glutamine concentration and achieved a reduction in skeletal muscle amino acid efflux. Wilmore's studies raised exciting prospects for applications in the clinical field. However, considering the known instability of free glutamine (Meister 1956; Stehle *et al.*, 1984; Stehle *et al.*, 1987) the scope for direct use of parenteral glutamine in TPN appeared to be limited. Subsequent studies in which continuous TPN with and without inclusion of the peptide Ala-Gln was given to catabolic rats (Fürst *et al.*, 1987) showed that in comparison with normal rats, injury induced a profound decrease in tissue free glutamine levels (plasma and liver ~20%, muscle ~45%) in animals receiving nutrition without dipeptide supplementation. Addition of the dipeptide to TPN significantly reduced the extent of the trauma-induced intracellular glutamine deprivation. On the other hand, the efficacy of treatment was rather moderate in the other tissues investigated. Thus, inclusion of Ala-Gln in the TPN resulted only in a slight increase in plasma free glutamine, while the elevation in liver free

Table 1.4 A comparison of recovered radioactivity in protein and non-protein fractions of various organs (tissues), following injection of ^{14}C-labelled L-Alanyl-L-glutamine (Ala-[U^{14}C]Gln) expressed as a percent of injected dose in rat and dog, respectively.

Organ (tissue)	Protein fraction		Non-protein fraction	
	Rat	Dog	Rat	Dog
Muscle	3.5 ± 0.35	3.0 ± 0.33	9.5 ± 1.10	10.4 ± 0.80
Liver	1.8 ± 0.02	2.0 ± 0.12	1.3 ± 0.20	1.5 ± 0.20
Kidney	0.3 ± 0.02	0.5 ± 0.03	0.2 ± 0.02	0.3 ± 0.03
Plasma	1.7 ± 0.10	1.8 ± 0.25	0.3 ± 0.04	0.4 ± 0.05
Mucosa	N. D.	7.6 ± 0.52	N. D.	0.6 ± 0.06
Serosa	N. D.	4.0 ± 0.30	N. D.	0.5 ± 0.03
Pancreas	N. D.	0.9 ± 0.07	N. D.	0.2 ± 0.01

N.D. = No data available

(From Fürst *et al.*, 1987)

glutamine pool was more apparent.

The results derived from isotope studies strongly indicate that the dipeptide Ala-Gln is easily available and rapidly used for protein synthesis, preferentially by muscle tissue. These findings in studies with continuous TPN offer firm evidence that parenterally administered Ala-Gln provides free glutamine especially for maintenance of the muscle free intracellular glutamine pool. Indeed, the discussion

should perhaps be focused on whether or not a normalization of the intracellular glutamine pool might promote protein synthesis during periods of catabolic stress: Rennie and colleagues (Rennie *et al.*, 1986; MacLennan *et al.*, 1987) were able to demonstrate a strong relationship between the synthetic rate of skeletal muscle protein and the intracellular concentration of free glutamine. Thus, it appears that maintenance of the muscle free glutamine pool might be a prerequisite for optimum protein synthesis in the catabolic state (MacLennan *et al.*, 1987).

1.9.3 Studies of L-Alanyl-L-Glutamine in Man

In considering results obtained from studies of glutamine metabolism in rats and their possible relevance to man it should be borne in mind that there is a marked difference in glutamine content between human and rat muscle. While glutamine represents more than 50% of the intracellular amino acid pool in human muscle, the major intracellular constituent in rat muscle is glycine (Fürst, 1985). Consequently, studies in man are necessary to demonstrate the influence of the dipeptide Ala-Gln on nitrogen economy in the catabolic state in man.

Utilization of Ala-Gln *in vivo* has been evaluated in healthy human subjects by kinetic studies and under conditions of continuous infusion of dipeptide supplemented amino acid solution (Albers *et al.*, 1986). The peptide elimination $t_{1/2}$ was 3.1 ± 0.16 min and that for the liberated free amino acids glutamine and alanine 8.2 ± 0.82 and 6.8 ± 0.34 min, respectively. During infusion of an amino acid solution supplemented with Ala-Gln and Gly-Tyr, the increments of plasma glutamine and tyrosine were 33 ± 2.2 % and 67 ± 5.7 % over the initial values. No peptide could be

detected in the urine. During the entire infusion period the dipeptide, though detectable in plasma, revealed only trace concentrations, suggesting virtually complete quantitative hydrolysis and utilization. In a subsequent study Brandl and colleagues (Brandl *et al.*, 1987) investigated *in vivo* utilization of a mixture of 5 glycyl-dipeptides and free amino acids administered to healthy volunteers over 12 hours. This study also demonstrated the efficient utilization of administered dipeptides in healthy man. Finally, provision of glutamine as the dipeptide (Ala-Gln) has been shown to increase glutamine availability, reduce post-operative muscle loss of alanine and glutamine and whole-body N-excretion (Stehle *et al.*, 1989), although no data are currently available to explain the likely mechanisms of this latter action in man.

1.10 Aim of the present studies

The aim of the studies detailed in this work was to clarify the role of the amino acid glutamine, provided as the dipeptide L-alanyl-L-glutamine (Ala-Gln), in modulating skeletal muscle and whole-body protein turnover in healthy postabsorptive man, and in patients undergoing major elective surgery when administered in supplementation to conventional non-glutamine containing intravenous nutrition. There has been considerable interest in this area of research during the last decade. To my knowledge however, no information is available on the role of glutamine in modulating muscle and whole-body protein turnover in healthy man. My initial hypothesis was that in healthy postabsorptive man, in whom the intracellular glutamine pool is not depleted, glutamine provision would

either have no significant effect, or possibly a small effect only, on muscle and whole-body protein turnover. I therefore began by designing a protocol to investigate the possible acute effects of parenteral glutamine provision, as the dipeptide Ala-Gln, on muscle protein synthesis and breakdown and whole-body protein turnover in healthy male postabsorptive subjects using standard stable isotope tracer techniques.

A number of workers have elaborated various facets of glutamine's role in maintaining skeletal muscle and whole-body protein and nitrogen balance in disease states such as sepsis, trauma and surgical injury. It appears from this body of work, undertaken mainly in animals but also to some extent in man, that depletion of the intramuscular free glutamine pool, occurring following injury, is associated with negative whole-body nitrogen balance and a reduction in skeletal muscle protein synthesis as determined by indirect semi-quantitative methodology. Provision of glutamine appears to reduce the extent of the post-injury depletion of the intra-muscular free glutamine pool and also appears to reduce the extent of the negative nitrogen loss. Although indirect methods of assessment, using the concentration of intramuscular polyribosomes as an index of protein synthesis, appear to indicate that this ameliorating effect of glutamine provision on post-injury nitrogen balance may occur as a result of a stimulation of protein synthesis, no direct evidence is available to explain the mechanisms of glutamine's action in human subjects. In these studies I have therefore set out to directly measure, using the same stable isotope methodology outlined above, the acute effects of conventional TPN, supplemented by Ala-Gln, on muscle protein synthesis and breakdown, and whole-body protein turnover, in patients undergoing elective major upper gastrointestinal surgery. My hypothesis was that in contrast to conventional

non-glutamine containing TPN provision, glutamine dipeptide supplementation in these patients would replete the intramuscular free glutamine pool and improve muscle protein balance. The study protocols were designed to make comparison firstly with the effect on muscle protein synthesis and breakdown (in the same patients) of provision of TPN supplemented by a control isonitrogenous solution of the free amino acids alanine and glycine. Further comparison was made with a different group of patients receiving TPN alone.

CHAPTER 2

MATERIALS AND METHODS

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2.1 The Use of Stable Isotopes in the Measurement of Protein Turnover

The measurement of protein turnover *in vivo* was shown to be possible in a series of pioneering experiments with ^{15}N and ^{2}H labelled amino acids initiated in the 1930's (Schoenheimer and Rittenberg, 1938). In the last two decades, there has been an increased impetus to investigate amino acid metabolism *in vivo* in man because of the availability of a range of commercially-produced stable isotope-labelled amino acids and the development of reliable and relatively inexpensive mass spectrometers by which to measure their labelling of trace compounds. Stable isotopes, as their name indicates, do not decay and do not release ionising radiation. These factors are of great importance in allowing their safe application in studies in neonates (Chien, 1991), children (Tomkins *et al.*, 1983), women in their reproductive years (Irving *et al.*, 1986) and young healthy subjects in general (Bennet *et al.*, 1989). When analysing stable isotope samples to determine the tracee molar enrichment only one measurement is required, whereas, the analysis of samples to determine radioactive tracee specific radioactivity requires separate measurements of radioactive and chemical concentrations, with the result that the coefficient of variation of the latter analysis is likely to be greater. In normal conditions, stable isotopes are administered as a tracer dose in <10% of the flux and the widely held assumption underlying the use of compounds containing stable isotopes that they behave exactly as the endogenous compound appears to be valid. A minor disadvantage is that these isotopes are ubiquitous and basal or background values (e.g. up to 1% for ^{13}C must be measured and taken into account. The stable isotopes most commonly used to investigate human protein metabolism are ^{2}H, ^{13}C, ^{15}N and ^{18}O. These stable isotopes have now been applied in a wide

range of metabolic studies of carbohydrate (McMahon *et al.*, 1989), fatty acids (Wolfe *et al.*, 1980) and protein metabolism (Bier, 1987; Halliday and Rennie, 1982; Wolfe, 1992) in man. They enable both qualitative and quantitative investigation of human metabolism at the whole body, tissue and substrate level.

2.2 L-[1-^{13}C]leucine as a Stable Isotope Tracer

Leucine represents approximately 8% of skeletal muscle protein (Sprinson and Rittenberg, 1949). The choice of leucine for the primed constant infusion method is theoretically sound because as opposed to other essential amino acids that are metabolised primarily in the liver, leucine is catabolised primarily in skeletal muscle, the body's principle protein reservoir (Young, 1970; Odessey and Goldberg, 1972). In addition, leucine appears to have a regulatory effect on the other two branched-chain amino acids, valine and isoleucine (Hambraeus *et al.*, 1976) and on muscle protein synthesis and degradation (Buse and Reid, 1975; Walser, 1984). It is also the main source of the alpha-amino nitrogen of gluconeogenic amino acids released from muscle (Odessey *et al.*, 1974; Adibi, 1976). Leucine is metabolised first by transamination to α-ketoisocaproate which is a reversible reaction and then by decarboxylation to isovaleryl-CoA and carbon dioxide (Figure 2.1). The carbon dioxide is released into expired air after first passing through the bicarbonate pool. In contrast to a ^{13}C label placed elsewhere in the leucine molecule, carboxyl-^{13}C is removed at the time when leucine is first irreversibly committed to oxidation, making L-[1-^{13}C]leucine an optimal, non-recycling tracer of the oxidation process of branched chain amino acids. The free

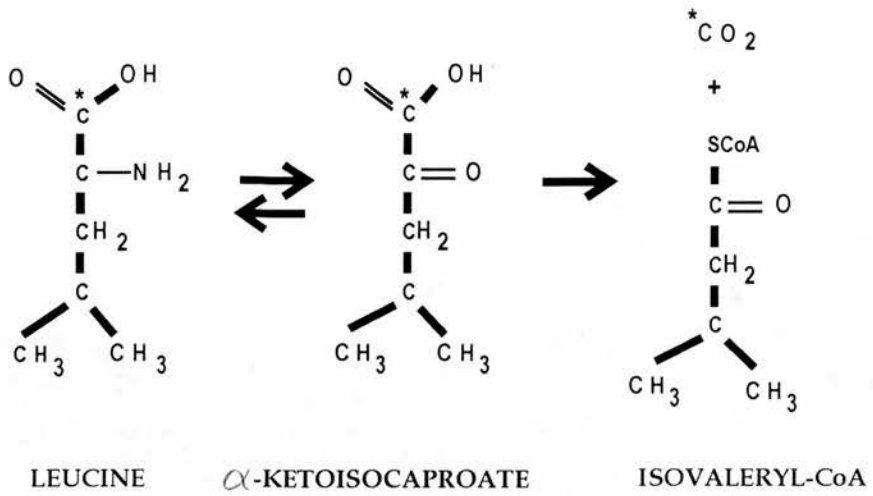

Figure 2.1 Leucine intermediary metabolism.

tracer, both in plasma and in intracellular fluid, is diluted by non-essential amino acids either released by protein breakdown or from the diet. The rates of these metabolic processes can be determined by the measurement of the ^{13}C enrichment of plasma α-ketoisocaproate and its oxidation product, carbon dioxide, excreted in breath and by comparison with the rate of infusion of tracer. The immediate metabolite of leucine, α-ketoisocaproate, is measured in preference to leucine on the assumption that this provides a closer measure of the tracer enrichment of leucine in the intracellular milieu (Matthews *et al.*, 1982; Rennie *et al.*, 1982b; Schwenk *et al.*, 1985a; Layman and Wolfe, 1987; Horber *et al.*, 1989.

TWO POOL MODEL FOR LEUCINE TURNOVER

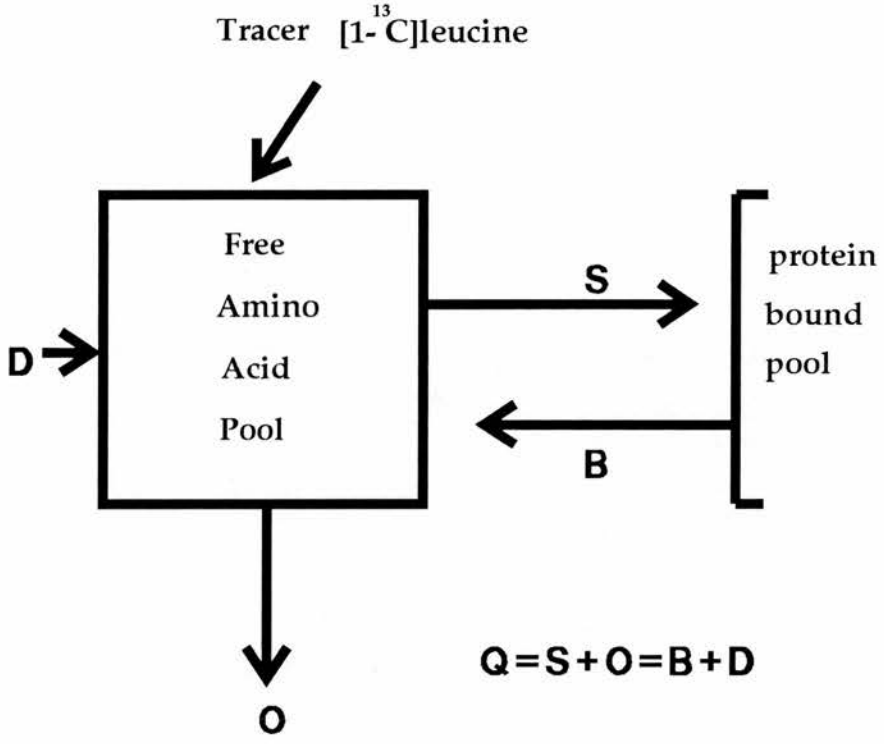

Figure 2.2 Two pool model of protein turnover; D = Diet; S = Synthesis; B = Breadown; O = Oxidation.

2.3 Whole Body Amino Acid Kinetics

The methodological basis of most apHxoaches to calculating *total protein* turnover is to measure the flux of an amino acid or of nitrogen (Waterlow and Stephen, 1967; Picou and Taylor-Roberts, 1969). The term *flux* refers to the rate of amino acid flow through the free amino acid pool into protein and other metabolic pathways (Figure 2.2). Amino acids enter the pool from the diet and from protein breakdown with additional (but quantitatively smaller) entry from *de novo* synthesis of the non-essential amino acids. The amino acids leave the pool by incorporation into protein (by protein synthesis), by oxidation and excretion and by certain other metabolic pathways e.g. in the formation of nucleotides and porphyrins.

The general procedure for measurement of flux is to label the free amino acid pool and to determine the amount of the label at subsequent times in a pool which can be sampled: for measurements of whole body turnover this would involve sampling of blood or urine. Flux can be measured either by using a *single dose technique* where a single injection of a radioactive tracer is given, knowing the amount of tracer given and dividing by the integral of the specific activity-time curve, or by using a *constant infusion technique* to attain plateau labelling by tracer in the free amino acid pool. When a labelled compound is administered and the specific activity in blood or urine is measured a specific activity-time curve is obtained showing an exponential fall in specific activity with time (represented schematically in Figure 1.4, Chapter 1, section 1.6.4.6). Stable isotope-labelled amino acids ([^{15}N]glycine, (Olesen *et al.*, 1954) and radioactive-labelled amino acids ([^{14}C]glycine, (Henriques *et al.*, 1955) have been used in this way to measure flux.

A simple two-pool model approach has been used in these studies to determine whole-body amino acid and protein turnover (Matthews *et al.*, 1980). In this model the whole body is considered as comprising two pools, a plasma pool and a protein pool. The flux (Q) for an amino acid is determined after a steady state is attained during a primed constant infusion of tracer amino acid and knowing the rate of infusion and measurement of the dilution of tracer in the plasma pool allows the calculation of the amino acid flux. The constant infusion method has the advantage that since the plateau is a straight line it can be defined by fewer points than are necessary for the specific activity-time curve after a single dose of tracer. Once the time required to reach the plateau value is known, no measurements need be made until plateau has been achieved. An important point to make with regard to the constant infusion method is that the plateau reached in this method is a 'pseudo'-plateau rather than a true plateau, in that the amino acids incorporated into protein are not irretrievably lost to the amino acid pool but there is in fact a slow return of tracer to it from the protein bound pool (by the process of protein degradation) resulting in a gradual increase in the specific activity in the infused pool. However, because of the much larger size of the protein bound pool in relation to the free amino acid pool, this return of tracer has been calculated to be slow enough such that a time of the order of 1000 hours would be required to significantly alter the free amino acid pool (Aub and Waterlow, 1970). In most experimental conditions therefore, where infusions last from 6-8 hours, the deviation of the 'pseudo'-plateau from the true is likely to be negligible.

Under steady state conditions, for essential amino acids such as leucine, flux may be calculated using the following equation:

$$\dot{Q} = \dot{S} + \dot{O} = \dot{B} + \dot{D}$$

Where Q is the rate of leucine flux or turnover, S is the rate of protein synthesis, O is the rate of amino acid oxidation (for leucine and valine, not phenylalanine), B is the rate of amino acid supplied from protein breakdown and D represents the dietary intake, all μmol/kg/h. In the fasted or postabsorptive state, dietary input is zero and therefore breakdown equals flux. If [1-^{13}C]leucine is used as tracer, the flux under steady state conditions may be calculated from the [1-^{13}C]leucine infusion rate and the extent either of leucine's labelling, or that of a metabolite (e.g. α-ketoisocaproate) taken to represent the precursor pool for protein synthesis:

$$\dot{Q} = i \times [\frac{E_i}{E_p} - 1]$$

Where i is the rate of tracer infusion (μmol/kg/h), E_i is the enrichment of the infusate (atom % excess or APE) and E_p is the plateau enrichment (APE) of the pool chosen to represent the precursor for protein synthesis i.e. arterial, venous or mixed venous plasma amino acid or α-ketoacid.

Plasma L-[1-^{13}C]leucine enrichment is measured by gas chromatography-mass spectrometry (Matthews *et al.*, 1980). Leucine oxidation may be measured in the whole body by means of collection of expired CO_2 with subsequent cryogenic purification and measurement of $^{13}CO_2$ enrichment by isotope ratio mass spectrometry (Halliday and Read, 1981). The rate of $^{13}CO_2$ released by leucine-

tracer oxidation (μmol/^{13}C kg/h) is:

$$F_{13_{CO_2}} = \frac{F_{CO_2} \cdot E_{CO_2}}{W} X \left[\frac{60 \cdot 41.6}{100 \cdot 0.95}\right]$$

where F_{CO_2} is the CO_2 production rate (cm^3/min^{-1})

E_{xO_2} is the $^{13}CO_2$ enrichment in expired air at isotopic steady state (APE)

W, the patient's weight (kg).

The constants 60 min/h and 41.6 μmol/cm (at S.T.P.) convert F_{CO_2} to μmol/h; the factor 100 changes atom % excess (APE) from a percentage to a fraction; the factor 0.95 accounts for the fraction of $^{13}CO_2$ released by L-[1-^{13}C]leucine oxidation, but not released from the body bicarbonate pool into expired air (Issekutz *et al.*, 1968; James *et al.*, 1976; Elia *et al.*, 1992). The rate of leucine oxidation is:

$$C = F_{13_{CO_2}} \left[\frac{1}{E_p} - \frac{1}{E_i}\right] X\ 100$$

from which the rate of leucine incorporation into protein (i.e. synthesis) can be calculated:

$$\dot{S} = \dot{Q} - \dot{C}$$

Finally, the rate of leucine release from the protein is the difference between leucine turnover and dietary intake:

$$\dot{B} = \dot{Q} - \dot{D}$$

When subjects are studied in the post-absorptive state,

$$\dot{D} = O,$$

and

$$\dot{B} = \dot{Q}$$

2.3.1 Assumptions Required for the two Pool Model of Leucine Metabolism

The assumptions required for the two pool model of leucine metabolism are:

1. Tracer (i.e. labelled) amino acid and unlabelled amino acid are metabolised in an identical form.
2. No re-entry occurs of labelled amino acid.
3. The tracer amino acid appears either in the free amino acid pool or bound in protein.
4. Enrichment in the plasma pool reflects the average intracellular enrichment.

2.3.2 Limitations of the Assumptions and Sources of Error

Some limitations of the assumptions and possible sources of error are:

1. *Non-homogeneity of the Free Amino Acid Pool*

   The model is based on the assumption of a single uniformly mixed free amino acid pool. This is in fact only partly true because of internal recycling due to dilution of the label in the intracellular pool with unlabelled amino acid derived from protein breakdown. This may lead to an error of up to 20% (Golden and Waterlow, 1977).

2. *Non-homogeneity of the protein bound pool*

   Although measurements and calculations are more straightforward, the basic two-pool model (Olesen, 1954) is probably over-simplistic and greater accuracy might be achieved by using more complex multiple-pool models (Cobelli, 1987).

3. *Re-entry of label*

   The calculation of flux assumes that there is no re-entry of tracer from labelled protein and this is not entirely correct.

4. *Routes of amino acid disposal*

   In calculating rates of protein synthesis from flux it is assumed that routes of disposal for amino acids are either by excretion or by incorporation into protein. However other routes of disposal are present such as the synthesis of nucleotides and amino acid sugars from amino acids; the formation of niacin from tryptophan and that of melanin from tyrosine and phenylalanine.

5. *Steady and non-steady states*

All calculations also assume a 'steady' state in which the amino acid pool sizes are constant throughout the period of investigation. In non-steady states with changes in pool size during the study period and where plateau is not attained equations originally developed by Steele (Steele, 1959) for use in studies of glucose metabolism with radio-active tracers and subsequently modified for use in stable isotope studies by Miles and colleagues (Miles *et al.*, 1983) should be used.

2.4 Determination of Whole-body Protein Turnover with [^{15}N]Phenylalanine: Equations for Steady State Conditions

The ^{15}N enrichment of plasma phenylalanine, during a primed continuous infusion of L-[^{15}N]phenylalanine under plateau conditions, is used to calculate the components of whole-body phenylalanine kinetics. A two pool model is employed with a) the free pool of phenylalanine in plasma and in cells and b) the protein-bound pool of phenylalanine (Figure 2.3). The equations are (Clarke and Bier, 1982):

$$\dot{Q} = \dot{B} + \dot{D}$$

where:

$\dot{Q}$ = the rate of phenylalanine turnover or flux

$\dot{B}$ = endogenous phenylalanine appearance or protein breakdown, and

$\dot{D}$ = dietary intake of phenylalanine.

The units of whole-body phenylalanine kinetics are micromoles of phenylalanine per kilogram total body weight per hour (μmol/kg/h)

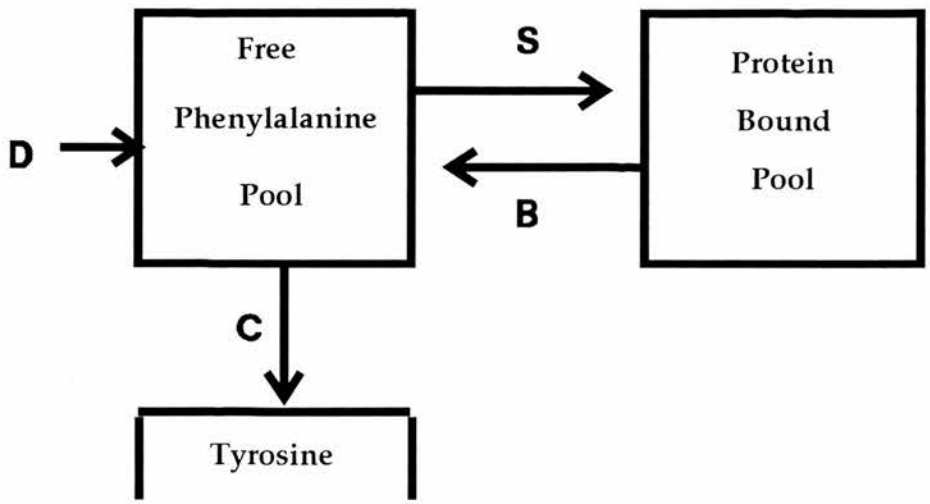

Figure 2.3 A two pool model of protein turnover with phenylalanine D = Diet; S = Synthesis; B = Breakdown; C = Catabolism to tyrosine.

The plasma phenylalanine flux is calculated with the equation:

$$\dot{Q} = i \left[\frac{E_i}{E_p} - 1 \right]$$

where:

i = infusion rate of L-[^{15}N]phenylalanine (μmol/kg/h),

Ei = ^{15}N enrichment of the infused L-[^{15}N]phenylalanine (APE), and

E_P = ^{15}N enrichment of plasma L-[^{15}N]phenylalanine (APE) after tracer equilibrium.

In the studies reported here a deep forearm antecubital vein was used as the sampling site. Ideally with phenylalanine the tracer would have been infused into the left heart and well mixed samples obtained from the right heart (Layman and Wolfe, 1987), a clearly impractical approach for studies in man. Infusion into a peripheral vein approximates a direct infusion into the left heart and sampling from a vein draining a large vascular bed may have enabled samples to be obtained which have been little different from right heart samples.

The first steps in the catabolism of phenylalanine involve hydroxylation of the aromatic ring, producing tyrosine. To calculate phenylalanine entry into protein an estimate of the rate of conversion of phenylalanine to tyrosine is required: this is calculated from simultaneous measurements of phenylalanine flux, tyrosine flux (with an additional tyrosine tracer), and appearance of the infused phenylalanine tracer in plasma tyrosine. In the studies reported here tyrosine flux and phenylalanine catabolism to tyrosine were not measured and, therefore, no estimate of non-catabolised phenylalanine disappearance (i.e. protein synthesis) can be made. Only whole-body total phenylalanine appearance and protein breakdown can be measured with the technique used. The equations for whole-body phenylalanine turnover are only valid where plateaux exist for both the ^{15}N enrichment and the concentration of plasma phenylalanine.

There are a number of assumptions underlying the use of these equations; these are:

a) The metabolism of ^{15}N-labelled phenylalanine is the same as that of unlabelled endogenous phenylalanine.

b) There is negligible recycling of tracer after it is incorporated into protein.

c) There are two pools of phenylalanine: a) free, both in the intravascular and intracellular compartments, and b) bound into proteins.

d) The degree of dilution of the label (the enrichment of phenylalanine in plasma) is representative of that of intracellular phenylalanine in the precursor pool for protein synthesis. Although this has not been confirmed in man it is shown in animal tissue *in vitro* and *in vivo*, for leucine at least, that leucyl-*t*RNA is charged with leucine coming predominantly from extracellular fluid or plasma (Davey and Manchester, 1969; Clark and Zak, 1981; Everett *et al*, 1981; Airhart *et al*, 1982). It is also known that phenylalanine readily enters into cells by the L-system transporter (used by leucine) and in addition, in liver by the T-system transporter. Neither of these transporters are modulated by hormones (Yudilevich and Boyd, 1987). It, therefore appears likely that tracer studies in man in which plasma phenylalanine enrichment is measured provide some measure of metabolism at an intracellular level. This approach awaits validation.

e) In subjects where phenylalanine was infused parenterally it entered into cells and mixed fully throughout the free phenylalanine pool.

2.5 Methods for Investigating Amino Acid and Protein Metabolism in Human Limb Tissues

Several techniques have been developed in the past two decades to measure limb muscle protein turnover. These include:

1. A semi-quantitative approach based upon comparison of the net amino acid

balance (or imbalance) and the extent of loss from muscle of the amino acid 3-methylhistidine, a specific index of myofibrillar protein breakdown (Lundholm *et al.*, 1982).

2. The use of stable isotopes to determine limb protein synthesis and breakdown by measuring the arteriovenous exchange of a labelled tracer amino acid (e.g. [^{15}N]phenylalanine) (Bennet *et al.*, 1990).

3. The measurement of protein synthesis directly by estimating the incorporation of stable isotope-labelled amino acids into muscle using muscle biopsy techniques (Bennet *et al.*, 1989).

2.5.1 Semi-Quantitative Approach using Amino Acid Efflux

The flux of an amino acid (either influx, i.e. uptake of amino acid or efflux, i.e., release of amino acid from muscle tissue) may be calculated from Fick's principle by multiplying the arteriovenous difference in concentration of an amino acid by the blood flow. Assumptions implicit in Fick's principle are that a steady state exists, that blood flow and metabolic rate are constant, that the blood flow in is equal to the blood flow out and that there is no interference from recirculation.

The amino acid tyrosine for example is not metabolised to any significant extent in skeletal muscle (Li *et al.*, 1973). Measurement of tyrosine arteriovenous difference correlates closely with net amino acid balance (Clowes *et al.*, 1980; Lundholm *et al.*, 1982; Bennegård *et al.*, 1984). The net amino acid balance (which is the difference between synthesis and breakdown of protein) can then be compared with efflux of a specific index of protein breakdown such as 3-

methylhistidine. 3-methylhistidine (i.e. N^{τ}-methylhistidine) is an amino acid produced by post-translational methylation at the N^{τ} position of histidine-73 of the actin chain (Vanderkerckhove and Webster, 1984). When released by the breakdown of myofibrillar proteins, 3-methylhistidine is not reincorporated into protein by protein synthesis and does not undergo intermediary metabolism (Young *et al.*, 1971). 3-methylhistidine is a constituent of actin (Asatoor and Armstrong, 1967) and the heavy chain of myosin in white muscle (Johnson *et al.*, 1967), proteins which together constitute 50% of muscle protein. Release of 3-methylhistidine, therefore, provides a specific index of breakdown of these myofibrillar proteins. It follows that factors modulating changes in the rate of breakdown of actin and myosin will induce changes in 3-methylhistidine release. In contrast, factors modulating changes in the rate of breakdown of mixed non-myofibrillar proteins may not cause any change in 3-methylhistidine release. Therefore, apparently conflicting results may be obtained from studies comparing changes in protein breakdown measured using 3-methylhistidine and for example tyrosine or phenylalanine release (Smith and Sugden, 1986). The release of 3-methylhistidine in urine has been widely advocated and used as an index of skeletal muscle protein breakdown *in vivo* (Ballard and Tomas, 1983). Objections to such use (Rennie and Millward, 1983) are based on the existence of 3-methylhistidine in non-muscle protein pools which turn over rapidly and contribute substantially to the total urine release of 3-methylhistidine (Clark and Spudich, 1977; Young and Munro, 1978; Emery *et al.*, 1986). For the rat, estimates of the source of total 3-methylhistidine from skeletal muscle, skin and gastrointestinal smooth muscle are 25%, 7% and 10% respectively; the source of more than half of the total urine excretion of 3-methylhistidine was not identified (Millward *et al.*,

1980c). For healthy man such information is not available. In sepsis however, in man, approximately 80% of excreted 3-methylhistidine derives from skeletal muscle with approximately 20% from non-muscle sources (Sjölin *et al.*, 1989). In studies of human protein metabolism seemingly directly conflicting results are reported with regard to the changes which may occur in urinary excretion of 3-methylhistidine (Rennie *et al.*, 1983). It is, therefore, likely that urinary excretion of 3-methylhistidine is not adequately specific for monitoring human skeletal muscle protein breakdown *in vivo* and only 3-methylhistidine efflux from the forearm has been employed as an index of skeletal muscle breakdown in the studies reported here. The major difficulty with the limb arteriovenous exchange technique is that the plasma concentration of 3-methylhistidine is very low (2-6 μmol/l) and can only be measured with sophisticated techniques such as automated ion exchange chromatography with fluorometric detection, used in the present studies.

The 3-methylhistidine efflux was calculated with the following equation:

$$Bal = (C_{A\text{-}3\text{-}mehis} - C_{V\text{-}3\text{-}mehis}) \cdot BF$$

Where:

$C_{A\text{-}3\text{-}mehis}$ and $C_{V\text{-}3\text{-}mehis}$ = plasma concentrations of 3-methylhistidine (μmol/l), and

BF = blood flow (ml/100g/min).

Units are nmol of 3-methylhistidine per 100g per min (nmol/100g/min).

Thus, in summary, a semiquantitative estimate of protein synthesis can be made from a comparison of the size and direction of change of tyrosine or phenylalanine balance and 3-methylhistidine output. No absolute rates of protein

synthesis can be arrived at however, although it is possible to make judgements about the direction and relative sizes of changes from normal in protein synthesis and protein breakdown.

2.5.2 Arteriovenous Phenylalanine Exchange by Limb Tissue Determined with L-[^{15}N]phenylalanine

This technique may be used to measure arteriovenous uptake and release of phenylalanine across a limb (Cheng *et al.*, 1985; Gelfand and Barrett, 1987; Thompson *et al.*, 1989), which predominantly contains skeletal muscle (Häggmark *et al.*, 1978). In skeletal muscle the only metabolic fates of phenylalanine are incorporation into protein and release from protein; catabolism of phenylalanine to tyrosine occurs predominantly in liver and does not occur in muscle. Uptake and release of phenylalanine by the limb are therefore, indices of protein synthesis and breakdown respectively.

The ^{15}N enrichment of arterial and venous plasma phenylalanine, during a primed continuous infusion of [^{15}N]phenylalanine, and plasma concentrations may be used to calculate phenylalanine exchange by limb tissue from the degree of dilution of the tracer in the venous outflow in comparison with the arterial inflow. The following equations (simplified from those of Cheng *et al.*, 1985) may be used (Gelfand and Barrett, 1987; Thompson *et al.*, 1989):

$$Bkdn = [\frac{E_{A-phe}}{E_{V-phe}} - 1] \cdot C_{A-phe} \cdot BF$$

where:

Bkdn = release of phenylalanine (protein breakdown) across the leg in nmol of phenylalanine per 100 g of leg tissue per minute (nmol/100g/min),

$E_{A\text{-}phe}$ = ^{15}N enrichment of arterial plasma phenylalanine (APE),

$E_{V\text{-}phe}$ = ^{15}N enrichment of venous plasma phenylalanine (APE),

$C_{A\text{-}phe}$ = arterial plasma concentration of phenylalanine in nmol/ml or μmol/l, and

BF = Blood flow of the limb in ml per 100 g limb per minute (ml/100g/min).

The net arteriovenous balance or the efflux of phenylalanine, a measure of protein balance, may be calculated with the following equation:

$$Bal = (C_{A\text{-}phe} - C_{V\text{-}phe}) \cdot BF$$

where:

Bal = arteriovenous balance of phenylalanine across the limb (nmol/100 g/min), and $C_{A\text{-}phe}$ and $C_{V\text{-}phe}$ = arterial and venous plasma phenylalanine concentrations (μmol/l).

In general the use of the term balance is preferred to the term flux. The former term was coined before the application of tracer techniques to the investigation of limb amino acid metabolism. The results with tracer techniques show that the flux of "flow" of amino acids is much greater than their respective net balances.

The arteriovenous uptake of phenylalanine or protein synthesis is calculated with the equation

Syn = Bal - Bkdn

where:

Syn = arteriovenous uptake of phenylalanine (nmol/100 g/min).

These equations are only applicable for a steady-state of concentration and ^{15}N enrichment of plasma phenylalanine.

There are a number of assumptions which are integral to the use of these equations. These are:

a) There are no differences between the metabolism of ^{15}N-labelled and endogenous phenylalanine.

b) There is negligible recycling of tracer after incorporation into protein.

c) Rapid exchange of phenylalanine occurs between plasma and muscle and, therefore, the ^{15}N enrichment of venous phenylalanine represents that entering protein synthesis within muscle, i.e. that of muscle phenylalanyl-tRNA.

d) The concentration of phenylalanine in plasma is the same as that of free phenylalanine within muscle.

e) When steady-state conditions are changed by a therapeutic action, complete exchange of phenylalanine occurs in the intramuscular free pool before further measurements are made.

f) The blood cells exchange amino acids rapidly and are involved in the across limb amino acid exchange. Pilot studies in our laboratory suggest that this is the case but exchange of tracer by the blood elements may be slower than that of plasma (K. Smith, personal communication).

g) The arterial blood flow is the same as the venous blood flow.

2.5.3 Calculation of muscle protein synthetic rate by determination of labelled amino acid incorporation into muscle protein

The method for the measurement of muscle protein synthesis using incorporation of a labelled amino acid is conceptually simple. A primed constant infusion of a stable isotope-labelled tracer amino acid is set up and muscle sampled by either a needle biopsy or conchotome forceps biopsy technique over a period of time when the plasma amino acid pool is labelled to a constant extent. The use of a priming bolus of tracer shortens the time required for the constant infusion (Matthews *et al.*, 1980). Ideally the rate of incorporation of label into protein should be measured by the comparison of two or more tissue biopsy samples taken at intervals after attainment of plateau labelling in plasma. However, in both patients and healthy volunteers, it is often not possible to obtain more than a single biopsy at the end of the period of infusion (i.e. at time t_2). Calculation then of incorporation (and synthetic rate) must be made from an assumed starting isotopic enrichment of mixed tissue protein (i.e. at time t_0), which can be obtained by measuring the mean L-[1-^{13}C]leucine enrichment in muscle from patients who have not been infused with L-[1-^{13}C]leucine (Gibson *et al.*, 1988). A further alternative exists for obtaining base-line values for [1-^{13}C]leucine enrichment of body protein against which to compare the incorporation of tracer [1-^{13}C]leucine into muscle protein. Since basal ^{13}C enrichment of leucine in whole blood proteins closely reflects the basal ^{13}C enrichment in protein-bound leucine in muscle (Heys *et al.*, 1990) a blood sample may be collected prior to the tracer infusion and mixed immediately with 2M perchloric acid for precipitation and later isolation and analysis, of whole-blood protein ^{13}C leucine enrichment (Heys *et al.*, 1990). This

was the method used in the present healthy volunteer studies where the local Ethical Committee approved one muscle biopsy only per study.

Muscle protein synthetic rates (k_s) can be calculated from the measured increase in incorporation of [^{13}C]tracer amino acid into the protein bound pool (ΔE_m) over a period of time (t_1, t_2) and the average enrichment of the pool chosen to represent the precursor pool for protein synthesis (E_p). For the primed constant infusion method the equation used was as follows (Rennie *et al.*, 1982a; Rennie *et al.*, 1982b):

$$k_s\ (\%/h) = \frac{\Delta\ E_m}{E_p} \times \frac{1}{(t_2 - t_1)} \times 100$$

where ΔE_m is the difference in enrichment between muscle biopsies or between a muscle biopsy and (basal) mixed whole blood protein

E_p is the average enrichment in the pool taken to represent the precursor and

t_1 and t_2 represent the time of biopsy (or, for t_1, sampling of whole blood).

2.6 Methods of measuring albumin turnover

In addition to studies of skeletal muscle and whole-body protein turnover, towards the end of these studies, as some plasma samples remained, the effect of Ala-Gln supplementation of conventional non-glutamine containing TPN was also investigated. A variety of methods have been employed to measure albumin turnover, including assay of the disappearance of ^{131}I-labelled albumin (Jensen *et*

al., 1967; Rossing, 1967) or incorporation of $^{14}CO_2$ (Tavill *et al.*, 1968). These methods have obvious limitations; they involve radioisotopes and require relatively long study periods (typically $\geq$ 12h). Stable isotope tracer methods have also been applied to the measurement of albumin turnover, initially using [^{15}N]glycine (Gersovitz *et al.*, 1980; Olefumi *et al.*, 1990; Yudkoff *et al.*, 1987). [^{13}C]leucine administered as a constant infusion (Carraro *et al.*, 1990; Olefumi *et al.*, 1990; Carraro *et al.*, 1990) or as a flooding dose (Ballmer *et al.*, 1990) has been used to investigate the effects on albumin synthesis of varying dietary protein (Carraro *et al.*, 1990), exercise (Carraro *et al.*, 1990), pregnancy (Olefumi *et al.*, 1991), diabetes and insulin administration (DeFeo *et al.*, 1991; Pacy *et al.*, 1990) and cirrhosis (Ballmer *et al.*, 1992).

Albumin synthesis rates obtained by the flooding dose (0.30%/h) and constant infusion (0.25%/h) are reportedly not very different when measured in separate studies by different investigators, when comparison has been made, and the size of any true difference could have been obscured (Ballmer *et al.*, 1990; Olefumi *et al.*, 1990). A recent study undertaken in our laboratory to directly compare the two methods suggests that the flooding dose method *per se* stimulates albumin synthesis (Smith *et al.*, 1993) and therefore should be considered carefully with regard to results obtained by its use in studies of e.g. nutritional intervention on albumin synthesis.

2.6.1 Calculation of albumin protein synthetic rate

In calculating albumin synthetic rate for greater accuracy it is advisable to

take into account the lag period between the synthesis and processing of albumin by the liver and its eventual secretion into plasma, i.e. the secretion time (t_s). The relationship between precursor and product is displaced by this secretion time, which can be calculated by plotting the regression line of the linear portion of the [^{13}C]leucine incorporation into albumin and extrapolating to the basal enrichment.

The rates of albumin synthesis were calculated by standard equations for the constant infusion method (Ballmer *et al.*, 1990; Garlick *et al.*,1989; Rennie *et al.*,1982). Albumin synthetic rate was calculated from the change in the measured ^{13}C enrichment of leucine in albumin divided by the area under the curve of precursor enrichment and was calculated after correcting for the lag period between infusion of label, its incorporation into albumin and its subsequent secretion from the liver. The secretion time (t_s) was calculated by extrapolation of the linear part of the curve of leucine incorporation into albumin to the base-line enrichment. The area under the curve of venous plasma α-ketoisocaproate (α-KIC) during each of the four hour periods of the split-period study was used as a measure of precursor enrichment during that period, i.e. in each case between 4 h of t_s and the time at which the end-of-period plasma sample was taken minus t_s. One criticism of using this approach is that the ketoacids are formed primarily in muscle, whereas albumin is synthesized mainly in the liver. However, this approach has recently been supported both by the closeness of the labelling of another branched-chain keto-acid, α-ketoisocaproate (αKIC), to that of the liver-derived very-low-density lipoprotein apolipoprotein B-100 (Pacy *et al.*, 1991) and by the similarity of albumin synthesis rate observed when the rate is calculated on the basis of αKIC and urea enrichment (Olefumi *et al.*, 1990).

2.7 CLINICAL METHODOLOGY

2.7.1 Subjects

Healthy volunteers were recruited from the staff of the Department of Anatomy and Physiology of the University of Dundee and the Department of Surgery of Ninewells Hospital and Medical School, Dundee. All healthy volunteers were screened by way of medical history-taking and none suffered from any acute or chronic illnesses and none were taking any medication. Pre-study screening blood tests for urea, creatinine, electrolytes, glucose and full blood count were normal. All subjects had stable body weights prior to study. There were no recent significant dietary changes and the diets were weight-maintaining.

Completed studies were performed in 16 patients who had undergone major elective abdominal surgery i.e. potentially curative oesophagectomy or oesophagogastrectomy for localised (as established by clinical examination, abdominal ultrasound and computerised axial tomography abdominal and thoracic scans) squamous or adeno-carcinoma of the lower third region of the oesophagus or of the gastro-oesophageal junction. They were provided with routine post-operative intensive care and parenteral nutritional support and were studied on the 3rd post-operative day. The expected age range of patients at the study outset, based on a review of hospital theatre records over the previous three years was 40-70 years. In order to minimise the effect of factors or variables known to affect muscle protein turnover, patients were excluded from the study if i) they had hormonal disorders (e.g. diabetes, thyroid disease), with the aim of avoiding the influence of these hormonal conditions on the protein turnover of patients studied

ii) metastatic neoplastic disease, to exclude the influence of cancer cachexia (Rennie *et al.*, 1983b) as a variable. All patients were allocated an APACHE II score (Knaus *et al.*, 1985) after an assessment carried out on routine post-operative admission to the intensive care unit, to provide an indication of the degree of pathophysiological disturbance resulting from the injury of surgery.

2.7.2 Ethical Approval

The studies reported in this work have been submitted for consideration by the Ethical Committee on Clinical Studies of the Dundee General Hospitals Group and formal approval has been obtained.

2.8 Adenocarcinoma of the Oesophagus

2.8.1 Pathology

The oesophagus is lined by stratified squamous epithelium down to the lower 2-3 cm. Ninety-nine percent of malignancies are squamous cell carcinomata. These are commonest in the mid-oesophagus just below the arch of the aorta. One percent of malignancies are adenocarcinomata. These arise in the lowest oesophagus either from an ectopic island of mucous epithelium which may occur just above the oesophago-gastric junction, or as a spread upwards of carcinoma of the cardia. Predisposing factors include oesophageal achalasia, a long-standing

structure, and the Plummer-Vinson syndrome (Watt and Spence, 1986). It is more common in smokers. It is likely that environmental factors are involved and the condition occurs more commonly in certain geographical areas (e.g. Brittany, Normandy and Transkei). The tumour typically spreads by submucosal invasion along the oesophagus. Lymphatic spread is either to the supraclavicular, subdiaphragmatic or mediastinal nodes. The oesophagus lies near vital neurovascular structures in the mediastinum which may be involved by local invasion. For these reasons effective surgical removal is difficult to achieve in other than early lesions, and the prognosis is usually poor.

2.8.2 Clinical presentation

The patient is usually over the age of 60. Lower third carcinoma are more common in males than females although the sex incidence for carcinomas of the middle and upper thirds is equal. The patient typically presents with a short history of progressive dysphagia. This initially affects solids only, but gradually increases until even swallowing liquids becomes a problem. Retrosternal pain may occur but is usually a late symptom. There may be regurgitation of food or fluids, or bloodstained vomiting. There is frequently a history of significant weight loss and the patient is usually cachectic. They may have palpable nodes and an enlarged liver but often, there are no abnormal signs.

2.8.3 Investigations

Barium swallow. Carcinoma of the oesophagus has a characteristic appearance of narrowing of the lumen which has an irregular, craggy, pitted surface and raised, rolled edges. The length of the narrowed lumen is used in staging.

Oesophagoscopy and biopsy. The carcinoma may be seen either as a malignant ulcer, a papillary growth or an irregular stricture. A biopsy is taken to confirm the diagnosis.

Oesophageal lavage and cytology. A cytological diagnosis of carcinoma may be made from cells isolated from oesophageal washings.

Computerized axial tomography. This gives a clear picture of the degree of mediastinal involvement.

These investigations aid in staging the extent of the disease and determining whether the tumour is potentially curable by local resection or not, and whether the patient is fit for major surgery. Of every 100 patients seen, about 60 will be considered operable. A final decision also takes into account the patient's general condition. The TNM (Tumour, Node, Metastasis) system of clinical staging is given in Table 2.1. In Western series the five-year overall survival is a disappointingly low 5% regardless of treatment. Prognosis appears to be related to size, especially length, and degree of spread at the time of treatment rather than to any histological feature, although adenocarcinomas tend to have a worse prognosis than squamous carcinomas.

TABLE 2.1 TNM (Tumour, Node, Metastasis) clinical staging of oesophageal carcinoma

Tis - Pre-invasive carcinoma (carcinoma-in-situ)
T0 - No evidence of primary tumour
T1 - Tumour involving 5 cm or less of the oesophageal length, producing no obstruction, does not involve the entire circumference of the oesophagus and which shows no evidence of extra-oesophageal spread
T2 - Tumour involving more than 5 cm of the oesophageal length and with no evidence of extra-oesophageal spread, or tumour of any size producing obstruction and /or involvement of the entire circumference of the oesophagus but with no extra-oesophageal spread
T3 - Tumour with evidence of extra-oesophageal spread, such as recurrent laryngeal, phrenic or sympathetic nerve involvement, fistula formation, involvement of trachea or bronchial tree, vena cava or azygos vein obstruction or malignant effusion

Cervical oesophagus

N0 - No evidence of regional lymph node involvement
N1 - Evidence of involvement of moveable unilateral regional nodes
N2 - Evidence of involvement of moveable bilateral regional nodes
N3 - Evidence of fixed regional nodes

Thoracic oesophagus

N0 - No evidence of regional node involvement on exploration or mediastinoscopy
N1 - Involved regional nodes

Distant metastases

M0 - No evidence
M1 - Presence of distal metastases

2.8.4 Forms of therapy

Oesophageal carcinoma may be treated by surgical resection, radiotherapy or palliative intubation or bypass. Squamous cell carcinoma can either be treated

by surgery or radiotherapy. Adenocarcinoma is not sensitive to radiotherapy.

2.8.4.1. Surgical resection

This is only attempted for growths confined to the oesophagus with no evidence of lymphatic or mediastinal spread. Of the 60 patients considered operable one third will be found to have more extensive tumours at the time of operation. Because of the likely presence of submucosal invasion curative resection requires wide excision of the oesophagus above and below the growth. The type of operation performed depends on the site of the growth (Table 2.2) (Dunn and Rawlinson, 1985).

Table 2.2 Carcinoma of the oesophagus: the sites of origin and the relevant surgical procedures.

Site	Procedure
1. Post-cricoid	1. Pharyngo-laryngo-oesophagectomy
2. Middle third	2. Total oesophagectomy
3. Lower third	3. Oesophago-gastrectomy

All the patients in the present studies had carcinoma of the lower third oesophagus or oesophago-gastric junction and oesophago-gastrectomy was the operative procedure performed. The stomach and lower oesophagus are mobilized

and the liver palpated. If the tumour is considered resectable, the chest is opened (left thoraco-abdominal incision) and the lower oesophagus including the tumour, all or part of the stomach, spleen and omentum are removed. The continuity of the oesophagus is usually restored either by anastomosis to the stomach remnant or, if all the stomach is resected, to a loop of jejunum.

2.8.4.2. Radiotherapy

This is used for squamous cell carcinoma in patients unfit for surgery or patients with a carcinoma involving the oesophagus above the arch of the aorta. There are few trials of radiotherapy performed for early carcinoma which would normally be considered resectable. The results appear to compare favourably with surgery although objective assessment cannot be made as the groups of patients are not comparable. Radiotherapy is also effective palliative treatment for dysphagia in some cases. Fifty per cent of patients need follow-up oesophageal dilatation. Complications include leucopenia, pulmonary fibrosis and spinal cord compression, all of which are rare with modern techniques.

2.8.4.3. Palliation

Of any 100 patients with oesophageal carcinoma, 60 will be found to have inoperable tumours and many of these will require some form of symptomatic relief. The most significant symptom is dysphagia. This may be relieved by i) intubation of the tumour, ii) a bypass operation iii) radiotherapy.

2.9 Experimental Environment

All subjects in the healthy volunteer studies attended the Department of Surgery on the day prior to the study. On arrival their weight, height and body fat composition - estimated from skin-fold thickness at four sites (Durnin and Womersley, 1974) - were determined. Informed written consent was obtained for participation in the study. The subjects re-attended the Department at 0645 h on the following day for the study. They remained recumbent throughout the study and the study room was maintained at a thermoneutral temperature between 25-27 °C.

In the patient studies the clinical background, including a review of the case records, of subjects who met the entry and exclusion criteria for the post-operative metabolic studies was discussed in detail with the clinician in charge of their care *prior* to their admission to hospital. All patients were approached on the day of their admission to one of the surgical wards in Ninewells Hospital and Medical School, usually the day prior to their operation. Informed written consent for participation in the metabolic studies was obtained and as with the healthy subjects, their weight, height and body fat composition were determined. The metabolic studies were carried out in the Intensive Care Unit, Ninewells Hospital and Medical School, on the third post-operative day. The subjects generally remained in a recumbent position throughout most of the duration of the study, excepting five to six brief periods of time (15-20 min each) when they received nursing care or chest physiotherapy.

2.10 MATERIALS

2.10.1 Tracers

All stable isotope labelled amino acids i.e., L-[1-^{13}C]leucine (99% ^{13}C), L-[^{15}N]phenylalanine (99% ^{15}N) and sodium [^{13}C]bicarbonate were purchased from Tracer Technologies Incorporated, Newton, MA, U.S.A. Immediately prior to administration the tracers were mixed in sterile pyrogen-free sodium chloride (NaCl) solution (Baxter Healthcare, Norfolk, U.K.) using an aseptic technique and the tracers were sterilised by passage through a 0.22 μm filters (Millipore SA, Molsheim, France). The tracer infusates were subsequently diluted to a total volume of 500 ml in sterile pyrogen-free NaCl solution (Baxter Healthcare, Norfolk, U.K.) and were infused *via* a second 0.22 μm filter.

2.10.2 Alanine, Glycine and Ala-Gln solutions

Solutions of the dipeptide Ala-Gln (Kabi-Pfrimmer, Erlangen, F.D.R.) and a mixture of the unlabelled amino acids L-alanine and glycine (Sigma Laboratories, U.K.) were prepared under aseptic conditions in sterile, pyrogen-free 0.9% (150 mmol/l) sodium chloride solution (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.). The solutions were sterilized by filtration through 0.22μm filters (Millipore SA, Molsheim, France). At the time of administration they also were infused *via* a second 0.22 μm filter.

2.10.3 TPN

From the first post-operative day, patients received parenteral nutrition consisting of a 50:50 mixture of two conventional commercial mixed amino acid solutions (Synthamin 9 and Synthamin 14, from Baxter Healthcare, Thetford, UK) providing 11.5 g nitrogen, Table 2.3) with 450 g glucose, 70 mmol Na^+ and 80 mmol K^+, all in 2.5 l water per 24 h. Patients received a 2.5 l bag of the solution over 24 h containing:

500 ml Synthamin 14
500 ml Synthamin 9 } (providing 11.5 g N/ 2.5 l bag)

1500 ml Glucose 30% (providing 7532 kJ)

70 mmol Na^+

80 mmol K^+

2.11 Blood sampling and infusion technique

In healthy volunteers, arterialised venous blood samples were taken from a 1.7 mm outer-diameter cannula (Venflon, Viggo AB, Helsinborg, Sweden), placed *retrogradely* in a dorsal hand vein and patency was maintained by a slow infusion of NaCl solution (250 ml over 8 h, 150 mmol/l). Before sampling, the hand was warmed for a minimum of 15 min by insertion into a thermostatically controlled chamber with a circulating air temperature of 75 - 80°C (Abumrad *et al.*, 1981). In the whole-body kinetics technique, it is essential to obtain mixed blood representative of that in all tissues, i.e., arterial blood, and the arteriovenous exchange technique also places a requirement for arterial samples. Arterial

Table 2.3 Composition of Synthamin Amino Acid Solutions

Amino acid	Synthamin 9 (g/l)	Synthamin 14 (g/l)
Alanine	11.38	17.60
Arginine	6.32	9.78
Glycine	5.66	8.76
Histidine	2.64	4.08
Isoleucine	3.30	5.10
Leucine	4.02	6.20
Lysine	3.19	4.93
Methionine	2.20	3.40
Phenylalanine	3.08	4.76
Proline	3.74	5.78
Serine	2.75	4.25
Threonine	2.31	3.57
Tryptophan	0.99	1.53
Tyrosine	0.22	0.34
Valine	3.19	4.93
Total L-Amino Acids	55.0	85.0
Total Nitrogen	9.1	14.0

cannulation however, used in research, poses unacceptable risks of complications to healthy subjects (Hall, 1971). The use of a heated superficial vein to obtain arterialised blood, the technique used on healthy subjects in the present studies, has been validated for obtaining arterial samples for blood gases and lactate (Forster *et al.*, 1972), glucose and alanine (Abumrad *et al.*, 1981) and ketone bodies (Sonnenberg and Keller, 1982).

A second 1.7 mm outer-diameter cannula was placed antegradely in an

ipsilateral antecubital vein of the forearm for infusion of amino acids, tracers and the dipeptide L-alanyl-L-glutamine. In the arteriovenous exchange studies, a third 1.7 mm. outer-diameter cannula was placed retrogradely in an antecubital vein of the contralateral forearm, to allow sampling of deep venous blood from the forearm. A paediatric sphygmomanometer cuff placed around the wrist was inflated to a pressure of 200 mmHg for 60 s before sampling to occlude the venous drainage from the hand. In composition, the hand proportionately has a much higher percentage of skin and bone and conversely muscle in comparison with the forearm and by excluding the hand circulation during venous sampling times, a greater portion of the venous drainage would come from the forearm, composed predominantly of muscle (about 75%). In man, bone accounts for about 10-12% of forearm volume: this tissue has a very slow turnover rate and bone blood flow was not recorded separately by the technique used in these studies. The remaining proportion of forearm, adipose tissue, has a low unit blood flow and contributes to, for example, phenylalanine metabolism to a negligible extent. It would appear therefore that muscle protein turnover predominantly accounts for the protein turnover of human limbs.

Blood gases (P_{CO_2}, P_{O_2}, SaO_2%) were measured routinely to ensure sufficient arterialisation i.e. >95% O_2 saturation was obtained and that deep venous blood was obtained from a site draining predominantly muscle, i.e. low O_2 saturation, <50% (Jackson *et al*, 1987), using a Corning 189 blood gas analyser (Corning Medical, Mass., USA).

In the studies involving postoperative subjects, arterial blood samples were obtained rather than *arterialised* venous samples, as these patients, studied in an intensive care unit (ICU) setting, had routine radial artery cannulation performed

by the anaesthetist in charge for the purpose of clinical monitoring of the patient's arterial blood gases. A cannula for sampling of venous blood was inserted retrogradely into an antecubital vein, as for the studies in healthy subjects. Patients also routinely had insertion of a triple-lumen cannula (KeyMed, UK), by a senior anaesthetist, into an internal jugular vein at the time of surgery, for subsequent use in monitoring central venous pressure in the 24-48 h postoperative period and for the infusion of intravenous drugs in this period. From the first post-operative day, one channel of the triple lumen cannula was used for the infusion of intravenous parenteral nutrition. On the third postoperative day, when the present studies were carried out, the second channel was used for the infusion of the study solutions (L-alanyl-L-glutamine, glycine *and* L-alanine). The third channel was used for infusion of tracer amino acids.

2.12 Blood Sample Handling

Blood samples were collected in tubes which contained additives appropriate for subsequent biochemical assays (Table 2.4). All blood samples were stored on ice prior to separation in a refrigerated centrifuge (4°C) at 1700 times gravity for 20 min. Samples were stored at -80°C until analysis. To prepare plasma for free amino acid concentration analysis, the plasma was deproteinised with sulphosalicylic acid (100 g/l, 1:4 v:v) containing norvaline internal standard (64 μmol/l), incubated for 30 min at 4°C to precipitate β-lipoproteins, passed through 0.45 μm filters before storage at -80°C. The perchloric acid extracts of blood for D-(-)-3-hydroxybutyrate analysis were neutralised by the addition of an alkaline

Table 2.4 Additives used in Blood Sample Tubes

Analysis	Additive	Quantity
Leucine Enrichment	Li Hep	
Phenylalanine Enrichment	Li Hep	
α-ketoisocaproate Enrichment	Li Hep	
α-ketoisocaproate Conc.	Li Hep	
Amino acid Conc.	Li Hep	
Glucose	Fluoride Oxalate	
D-(-)-3-hydroxybutyrate Conc.	Perchloric acid	1:1 v/v
Insulin	Li Hep	
C-Peptide	Li Hep	
Glucagon	EDTA-Aprotinin	500 KIU/ml
Insulin-like Growth Factor 1	EDTA	
Packed Cell Volume	Li Hep	
Blood Gas Conc.	Li Hep (syringe)	
Blood CO_2 Enrichment & Conc.	Orthophosphoric acid	

Abbreviations: Li Hep, lithium heparin; EDTA, ethylenediaminetetra-acetic acid; KIU, kallikrein inhibitor units.

solution containing 2 mol/l KOH, 0.4 mol/l KCl and 0.4 mol/l imidazole before storage at -80°C. Plasma for hormone analysis e.g. insulin and glucagon, was aliquoted separately into EDTA tubes and stored at -80°C until analysis. To treat and store blood for subsequent determination of whole blood carbon dioxide concentration and ^{13}C enrichment, the blood (1 ml) was added to 20 ml glass evacuated tubes (Vacutainer, Becton Dickinson, NJ, USA) immediately on sampling. Before use these tubes were prepared by adding 1 ml of a mixture of orthophosphoric acid (6 mol/l) and silicone antifoam (10% by volume) and the tubes

were degassed to a pressure of 0.4 mbar and sealed (Read *et al.*, 1984).

2.13 Breath Sampling and Indirect Calorimetry

Before tracer administration and at intervals throughout the healthy volunteer studies expired breath was collected into 2 l latex bags and an aliquot was transferred into 20 ml evacuated glass tubes (Vacutainer) for storage and subsequent determination of ^{13}C enrichment in carbon dioxide, as detailed below. Total carbon dioxide production and oxygen consumption was determined over the 8 h period by two separate 10 min collections of expired breath into a 100 litre Douglas bag, the volume being determined by a Harvard dry gas meter. Samples of the breath (200 ml) were subsequently analyzed for CO_2 with an infra-red absorption CO_2 gas analyzer (Analytical Development Co., Ltd., U.K.). In patients, the protocol requirements and the arrangements for expired breath collections and indirect calorimetry were initially identical, although in a large number of patients, because of technical and other limitations described in Chapter 5, the protocol requirements could not be met.

2.14 Muscle Biopsy Technique

Anterior tibial muscle was biopsied using 6.5 mm Tilley Henckel ethmoid punches (S. Murray and Co., Sheffield, U.K.) via a 10 mm long skin incision made after 5 ml of 20 g/l lignocaine local anaesthetic was infiltrated superficially to the

fascia (Dietrichson *et al.*, 1987). At each time point one to three biopsies were obtained providing 100 to 300 mg wet weight of muscle. The biopsies were from the fullest part of the shin and were taken 10 mm deep to the fascia. After each biopsy firm pressure was applied for 10 to 15 min over the site, and the skin incision was held closed with surgical tape. In the patient studies a second biopsy was taken from either the same leg, at a distance of 40 mm to the first biopsy, or from the contralateral leg. A third biopsy was taken from the leg contralateral to that first biopsied. The muscle samples were immediately frozen in liquid nitrogen and stored at -196°C until analysis.

2.15 Limb Blood Flow

Blood flow was measured in the study forearm using venous occlusion strain gauge plethysmography using a commercially available instrument (Medimatic, Denmark) regularly maintained and calibrated by the Vascular Laboratory, Ninewells Hospital and Medical School, Dundee and kindly lent for the present metabolic studies. Appropriate precautions were taken according to guidelines outlined by Jackson *et al.* (1987) with, in particular, a paediatric wrist cuff being applied, as described above, prior to blood flow measurement in order to stabilise the measured blood flow by minimising the variable effects of the cutaneous circulation on flow. The technique of mercury-silastic strain gauge plethysmography (Whitney, 1953; Needham, 1972; Englund *et al.*, 1972; Sumner, 1985) has been shown to give reproducible results (Roberts *et al.*, 1986) and to show appropriate blood flow changes resulting from exercise (Hughson, 1988).

2.15.1 Tracer Infusions

At the start of tracer infusion doses of L-[1-^{13}C]leucine (1.0 mg/kg) and sodium [^{13}C] bicarbonate (either 0.16 for volunteer or 0.24 mg/kg for patient studies) were administered to prime their respective body pools (Allsop *et al.*, 1978; Matthews *et al.*, 1980; Nair *et al.*, 1988a). A continuous infusion of L-[1-^{13}C[leucine at a rate of 1 mg/kg/h was administered throughout the studies. This was diluted to a volume of 500 ml in sodium chloride (150 mmol/l NaCl), and was delivered at a rate of 62.5 ml/h with IMED 928 volumetric infusion pumps (IMED Ltd, Abington, U.K.). In the arteriovenous exchange studies priming doses of L-[^{15}N]phenylalanine (0.5 mg/kg) were administered, followed by a continuous infusion (0.5 mg/kg/h) throughout the studies.

2.15.2 Ala-Gln and Ala + Gly infusions

In the healthy volunteer studies, control subjects received an 8 h constant infusion of alanine and glycine (Kabi-Vitrum, Sweden) at 4.8 mg/kg/h and 8.0 mg/kg/h respectively (after priming bolus doses of 4.8 mg/kg and 8.0 mg/kg) providing a total of 2.25 mg amino-N/kg/h. The study group received a primed constant 8 h infusion of L-alanyl-L-glutamine (Kabi-Vitrum, Sweden) at 11.7 mg/kg/h, also providing 2.25 mg amino-N/kg/h, after a priming dose of 11.7 mg/kg. The solutions were delivered by an IMED 928 volumetric mixed infusion pump (IMED Ltd, Abington, U.K.). In the longitudinal patient study (see below for

description of protocol), a mixed solution of alanine (4.8 mg/g/h) and glycine (8 mg/kg/h) were infused in the first 4 h period in amounts isonitrogenous with the alanyl-glutamine (11.7 mg/kg/h) given in the second 4 h period, when the alanine and glycine were discontinued. Priming doses were given of the alanine and glycine mixture and the alanyl-glutamine solution in the first 10 min of each 4 h period respectively, the priming doses providing the same dose of alanine and glycine or alanyl-glutamine as the amount infused during each hour of the subsequent continuous infusion.

2.16 PROTOCOLS

2.16.1 Protocol 1: Healthy Volunteer Studies

This protocol (Figure 2.4) was designed to investigate the acute effects on skeletal muscle protein synthesis and breakdown of the dipeptide L-alanyl-L-glutamine administered parenterally as a continuous infusion to healthy volunteers. Standard stable isotope methodology was used with administration of a primed constant infusion of tracer ([1-^{13}C]leucine and [^{15}N]-phenylalanine) and measurements of tracer ([1-^{13}C]leucine) incorporation into muscle protein, to determine protein synthesis, and measurements of arteriovenous dilution of tracer ([^{15}N]-phenylalanine) to determine protein breakdown and synthesis over an 8 h period. In an analogous manner to a previous clinical study in post-operative patients (Stehle *et al.*, 1989), an isonitrogenous solution of glycine and alanine was used as the control solution. All the subjects consumed a weight-maintaining diet

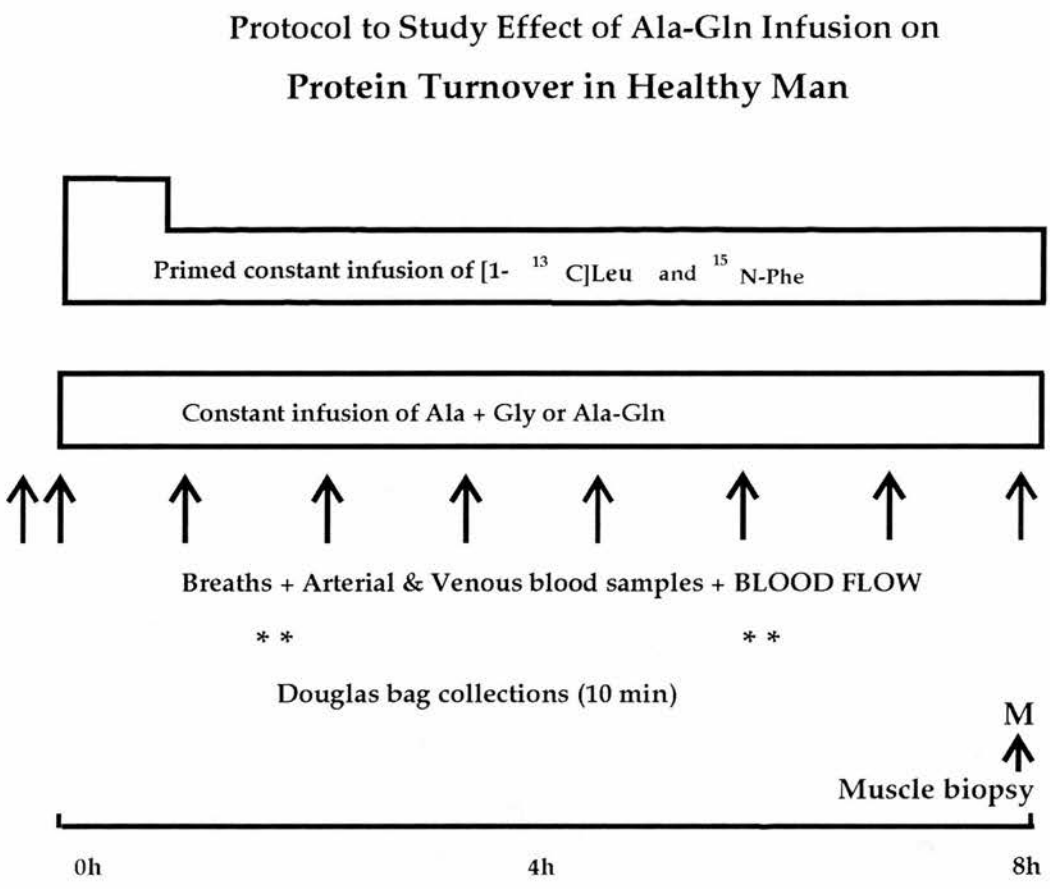

Figure 2.4 Protocol 1: for the study of healthy subjects.

during the week before the study. They took their evening meal between 5-30 and 7 pm, on the evening prior to the study. Thereafter they consumed no food by mouth until completion of the study on the following day; only water was given. The study was carried out between 7 am and 4 pm.

2.16.2 Protocols 2 and 3: Patient Studies

These studies were designed to investigate the effect of conventional total parenteral nutrition (TPN) on muscle protein synthesis and breakdown in post-operative subjects (i.e. receiving TPN alone) and subsequently to investigate the acute effects on protein synthesis and breakdown of alanyl-glutamine supplementation of the conventional TPN. All patients were provided with routine post-operative intensive care and nutritional support. The patients were studied on their 3rd post-operative day. From the first post-operative day, all patients received TPN and this was continued at least up to and including the third post-operative day when the tracer study was carried out. On the third post-operative day, during the course of an 8 h [1-^{13}C]leucine and [^{15}N]-phenylalanine infusion, the incorporation of tracer into skeletal muscle protein and the arteriovenous dilution of tracer across the forearm was measured using two different protocols. The first patient study protocol, a 'cross-sectional' control study of Ala-Gln supplementation, Protocol 2 (Figure 2.5) was used on a 'pilot' basis to obtain information on the effect of conventional TPN on skeletal muscle protein synthesis and breakdown in these patients, who had undergone major surgery, by direct (tracer incorporation into muscle protein, to determine protein synthesis) and

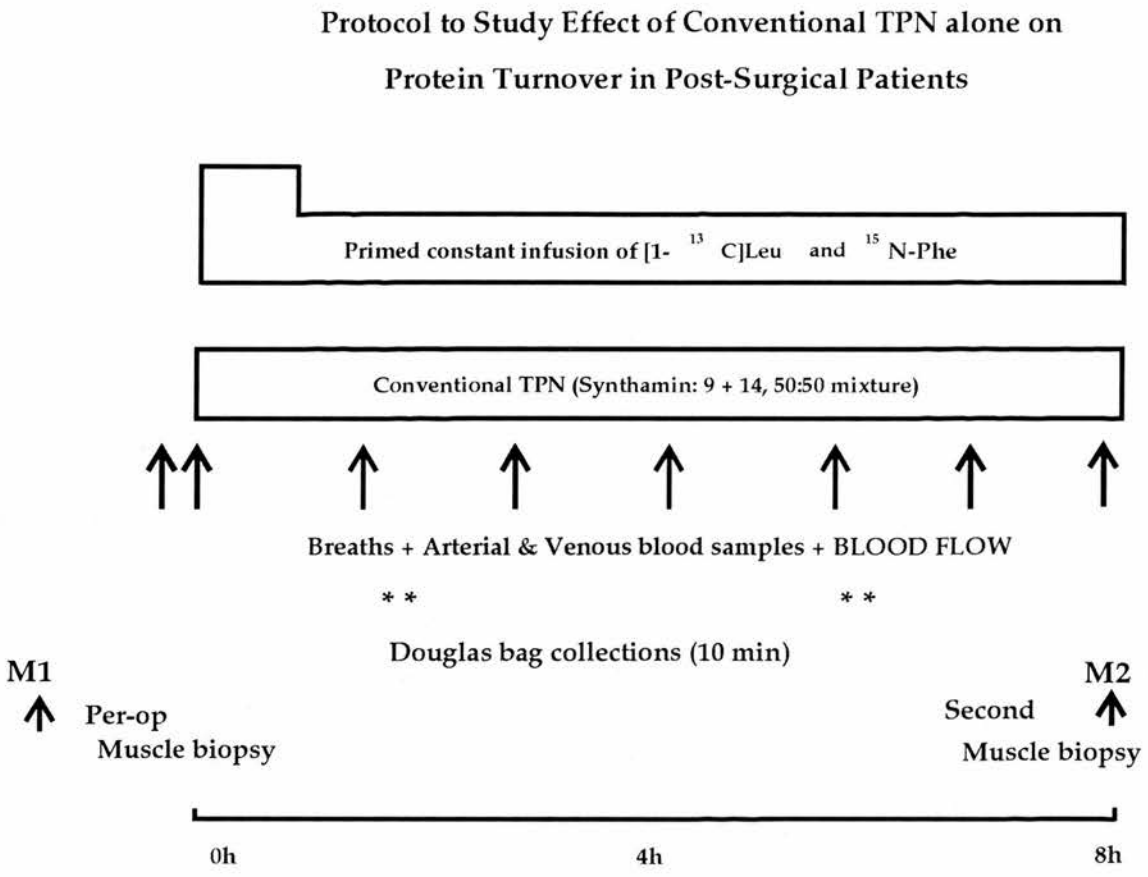

Figure 2.5 Protocol 2: for the study of post-operative patients receiving TPN alone.

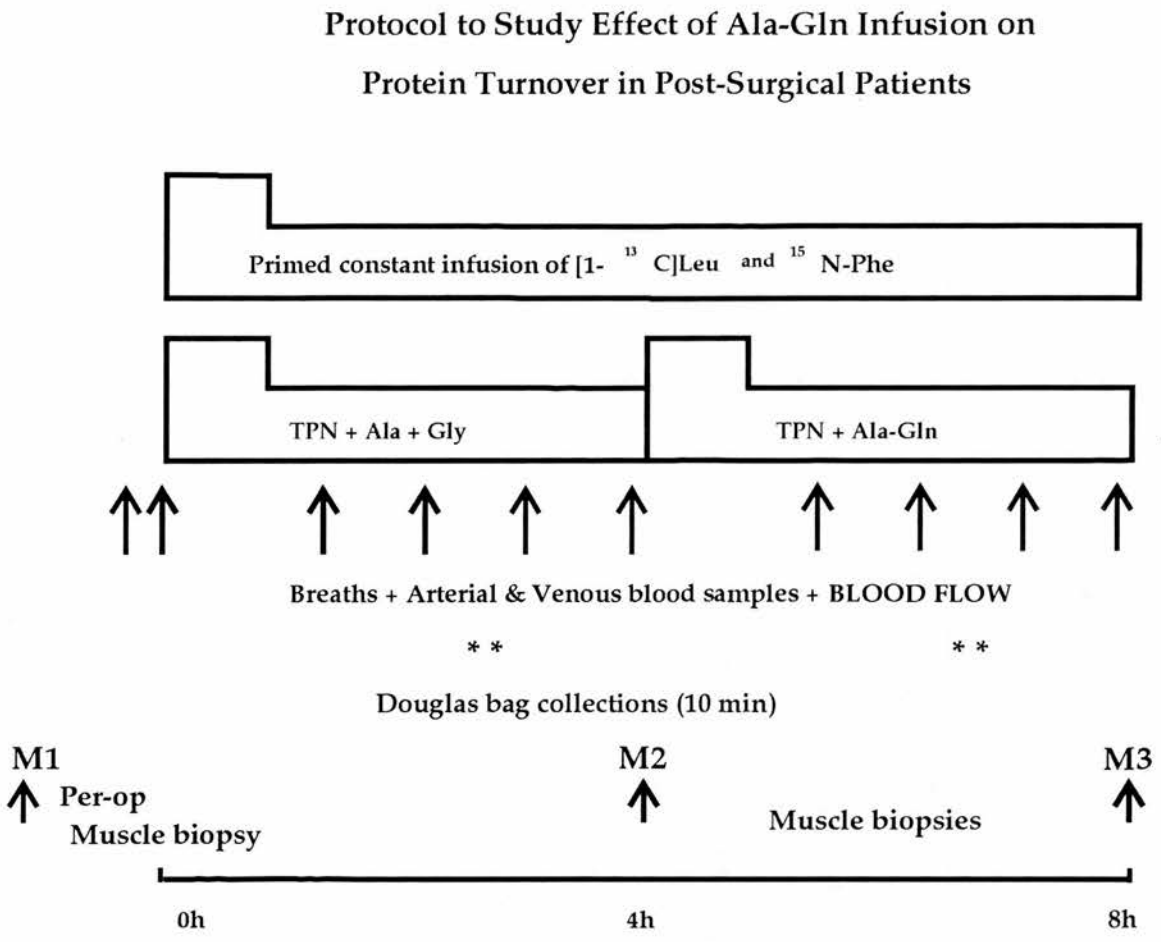

Figure 2.6 Protocol 3: for the study of post-operative patients receiving TPN supplemented by either Ala+Gly or Ala-Gln.

indirect (arteriovenous dilution of tracer to determine protein breakdown and synthesis) measurements over an 8 h period.

The second patient study protocol, a 'longitudinal' study of Ala-Gln supplementation, Protocol 3 (Figure 2.6), required measurement of the incorporation of[1- ^{13}C]leucine into muscle protein and arteriovenous dilution of [^{15}N]-phenylalanine during two 4 h periods: firstly the conventional TPN solution was supplemented with alanine (4.8 mg/kg/h) and glycine (8 mg/kg/h) in amounts isonitrogenous with the alanyl-glutamine (11.7 mg/kg/h) given in the second 4 h period, when the alanine and glycine were discontinued. Priming doses of the alanine and glycine mixture and the alanyl-glutamine solution were infused over the first 10 min of each 4 h period respectively: these priming doses provided the same dose of alanine and glycine or alanyl-glutamine as the amount infused during each hour of the subsequent continuous infusion. This protocol produced steady-states for both the plasma leucine and phenylalanine concentration and the ^{13}C enrichments of leucine, α-ketoisocaproate and ^{15}N enrichment of phenylalanine about 30-60 min after commencement of the respective study solutions. The TPN solution, the tracer solution and the study solutions were infused at a constant rate by separate IMED 928 volumetric infusion pumps.

2.17 LABORATORY ANALYSIS

2.17.1 Gas Chromatography-Mass Spectrometry methods for the determination of enrichment and concentration

The concentration and enrichment of the tracer amino acids and keto-acids

were determined by gas chromatography-mass-spectrometry (GC-MS). The ^{13}C enrichment of the amino- and keto-acids was determined by monitoring a specific fragment (or m/z), containing the labelled moiety in the mass spectrum of each amino- or keto-acid (see Table 2.5). In each case the [^{13}C] label is revealed as 1 atomic unit (amu) higher than the unlabelled compound. This distinction permits the determination of the amount of labelled material present via changes in the $(M+1)^+$ to $(M)^+$ ratio i.e. ^{13}C:^{12}C. The isotope ratio of ^{13}C to ^{12}C is calculated from the integrated area under the peak from both masses. Comparison of this ratio before and during infusion of tracer allows determination of the enrichment of the compound of interest and may be calculated from the equation:

$$Enrichment\ (APE) = \frac{R_S - R_B}{1 + (R_S - R_B)} \times 100$$

where, APE = atoms percent excess

R_s = isotope ratio of enriched sample, and

R_B = isotope ratio of unenriched or basal sample

The concentration was determined by the addition to plasma, before the addition of urease, of the internal standards L-[methyl-2,4,5-^{13}C]leucine, L-[ring-$^{2}H_5$]phenylalanine and α-[5,5,5-$^{2}H_5$]ketoisocaproic acid, at approximately 5-10% of the concentration of tracee (endogenous amino- of keto-acid) expected in plasma. The ratio of the peak area of amino acid or ketoacid to the internal standard i.e.

Table 2.5 Ratios of mass to charge (m/z) monitored for the determination of tracer enrichment and concentration.

	$(M)^+$	$(M+1)^+$	Internal Standard* $(M+X)^+$
Amino acids			
Valine	288	289	291
Leucine	302	303	305
Phenylalanine	336	337	341
α-KIC	259	260	262

* where X equals the number of mass units each internal standard is above the naturally occurring compound e.g. *ring*-$d_5$phenylalanine = $(M+5)^+$ or 234+5 = 239.

$(M)^+ + (M+1)^+/(M+X)^+$ was then compared with a standard curve containing known amounts of standard relative to the internal standard prepared and treated identically to the plasma samples.

Gas chromatography-mass spectrometry was performed on one of two instruments: a Finnigan 1020 GC-MS (Finnigan MAT, Hemel Hempstead, UK) or an HP 5971A MSD (Hewlett Packard, Cheadle Heath, UK) operated in the selected-ion-monitoring (SIM) mode, using electron-impact (EI) ionization (70 eV). The column (OV-1 type, 15 m x 0.32 mm i.d.) and operating conditions employed were similar for both instruments and for both amino and ketoacid derivatives.

The initial column temperature, for amino acid separation, was 140°C which was held for 1 min before being increased to 280°C at 20°C min^{-1} where it was held for 3 min prior to cooling. The injector temperature was 250°C; injection was

performed in the splitless mode and the transfer line was maintained at 280°C. The carrier gas was helium (BOC, UK) and the column head pressure was set at 5-7 kPa. Conditions for keto-acid separation differed slightly in that the column temperature programme was increased by 15°C min^{-1} to 260°C and the injector temperature was 240°C. This allowed better separation and peak symmetry of the keto-acid quinoxalinol-*t*-BDMS derivatives.

Table 2.6 Determination of Isotope Ratio of Plasma Leucine and Phenylalanine

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This analysis was based on the method of Mawhinney *et al.*, (1986) and employed a t-butyldimethylsilyl derivative, separation by gas chromatography and isotope quantification by mass spectrometry in the electron impact mode.

Method

1) To a microcentrifuge tube add 600 μl of plasma and 20 μl of urease solution (Urease from jack bean, Type IX, 500 units/ml in 0.01 mol/l phosphate buffer, pH 6.7 with sodium azide 1 μmol/1), vortex to mix and incubate at 37°C for 15 min. For samples where high leucine and phenylalanine concentrations are anticipated the initial volume should be appropriately reduced.
2) To precipitate the proteins, add 700 μl methanol/HCL (4:1 v/v), vortex to mix and centrifuge at high speed for 5 min.
3) Decant supernatant to a screw top microcentrifuge tube and remove the solvent by overnight rotary-evaporation.
4) Add 200 μl pyridine and 50 μl N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide, cap tightly and vortex.
5) Incubate at 80°C for 60 min total; remove from the oven after 30 min, vortex to mix and return to the oven.
6) Add 400 μl decane, vortex and centrifuge for 3 min at high speed. Transfer to autosampler vials

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2.17.2 ^{13}C Analysis of Plasma Leucine

1-^{13}C Enrichment of leucine in plasma was determined after extraction of amino acids and preparation of *tert*-butyldimethylsilyl derivatives by reaction with N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (Regis Chemical Company, Morton Grove, IL, U.S.A.) (Mawhinney *et al.*, 1986). (Table 2.6). The [1-^{13}C] enrichment in leucine was determined by monitoring ion currents m/z 302 and 303. The retention time for this derivative of leucine was 3.7 to 3.8 min.

2.17.3 ^{15}N Analysis of Phenylalanine

The preparation of plasma phenylalanine samples for ^{15}N enrichment analysis by gas chromatography-mass spectrometry was identical to that used for leucine (Table 2.6). The ion currents m/z 336 and 337 were monitored. The retention times for the phenylalanine derivative were in the range of 5.6 to 5.8 min (i.e. longer than those for leucine). The analysis of leucine and phenylalanine was normally carried out on the same sample.

2.17.4 ^{13}C Analysis of Intramuscular Free Leucine

The ^{13}C enrichment of intramuscular free leucine was determined in neutralized perchloric acid (0.5 mol/l) extracts of muscle, ground to a fine powder in liquid nitrogen (-196°C), using a pre-cooled mortar and pestle. The samples were

purified using cationic ion exchange-chromatography (Table 2.7). *t*-Butyldimethylsilyl derivatives were prepared and gas chromatography-mass spectrometry was carried out a minimum of two times on each sample.

Table 2.7 Determination of Isotope Ratio of Intramuscular Free Amino Acids

This analysis was also based on the method of Mawhinney *et al.* (1986), and employed a t-butyldimethylsilyl derivative, separation by gas chromatography and isotope quantification by mass spectrometry in the electron impact mode.
Method

1) A sample of muscle (about 100-300 mg) is ground to a fine powder in liquid nitrogen with a pre-cooled mortar and pestle with removal of any obvious pieces of connective tissue. An aliquot (approximately 20 mg in weight) of the finely ground powder was taken for estimation of free amino acid concentrations, extracted with ice-cold perchloric acid (PCA).
2) Extract free amino acids by adding 3 ml perchloric acid (0.5 mol/l, with norvaline 4 μmol/l to quantify amino acids using the amino acid analyser), vortex and stand in ice for 30 min.
3) Centrifuge for 20 min (3000 RPM, 4°C) and decant supernatant. The isotope ratio of protein-bound leucine is determined in the residue (Table 2.8).
4) To 500 μl of the supernatant add one drop of universal pH indicator and neutralise with potassium bicarbonate (2 mol/l). Vortex, let stand in ice for 20 min and vortex again.
5) Centrifuge (3000 RPM, 20 min, 4°C) and pipette off supernatant. Mix with an equal volume of glacial acetic acid. Apply to a 2.5 ml bed of ion-exchange resin (Dowex 50W-X8(H^+), 100-200 mesh), prewashed in HCL (1 mol/1).
6) The column is washed with HCL (2x1 ml) and then distilled deionized water (2x1 ml).
7) The amino acids are eluted with 3 ml of ammonia solution (4 mol/1) and are collected in a screw-top microcentrifuge test tube.
8) Remove the ammonia by overnight rotary-evaporation.
9) Add 50 μl pyridine and 50 ul N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide, cap tightly and vortex.
10) Incubate at 80°C for 60 min total; remove from the oven after 30 min, vortex to mix and return to the oven.
11) Add 50 μl decane, vortex and centrifuge for 3 min at high speed. Transfer to autosampler vials.

Ketovalerate was included as internal standard to quantify the α-ketoisocaproate.

2.17.5 ^{13}C Analysis of Plasma α-Ketoisocaproate

To determine the ^{13}C enrichment of plasma α-ketoisocaproate, *o*-trimethylsilyl quinoxalinol derivatives were prepared using *bis*-(trimethylsilyl)trifluoroacetamide (Regis Chemical Company or Sigma Chemical Company Ltd, Poole. U.K.) (Table 2.8) (Rocchiccioli *et al.*, 1981). Gas chromatography-mass spectrometry was by analogous methods to those used for plasma amino acid analysis with monitoring of ion-currents m/z 259 and 260.

2.17.6.1 Isotope Ratio Mass Spectrometry

The enrichment of ^{13}C in carbon dioxide was measured with an automated Finnigan MAT breath gas analysis system attached to a Finnigan Delta D isotope ratio-mass spectrometer.

2.17.6.2 ^{13}C Analysis of Carbon Dioxide in Breath and Blood

Carbon dioxide in breath was cryogenically purified on-line before measurement of enrichment (Scrimgeour and Rennie, 1988). Analysis of ^{13}C enrichment of carbon dioxide in whole blood was performed with the same equipment after liberation of the carbon dioxide with orthophosphoric acid. The total blood concentration of carbon dioxide was measured at the same time by comparing the pressure of the carbon dioxide released from the blood into the

Table 2.8 Determination of Isotope Ratio of Plasma α-Ketoisocaproate

This analysis used the method of Rocchiccioli *et al.*, (1981), further modified by Ford *et al.*, (1985), employing a quinoxalinol derivative, separation by gas chromatography and isotope quantification by mass spectrometry in the electron impact mode.

Method

1) To a microcentrifuge tube add 600 μl plasma, 30 μl ketovaleric acid solution (0.1 mg/ml) and 800 μl ethanol to precipitate the protein. Vortex to mix.
2) Centrifuge at high speed for 3 min, decant the supernatant to a test tube and dry under a stream of nitrogen at 50°C.
3) To the residue add 200 μl distilled water and 100 μl *o*-phenylenediamine solution (2% w/v in HCL 4 mol/l).
4) Heat in the oven for 60 min at 90°C, then cool.
5) Extract with 2 by 1 ml aliquots of ethylacetate and dry the combined extracts over anhydrous sodium sulphate.
6) Decant the dried ethylacetate to a reaction vial, taking care not to transfer any sodium sulphate. Dry under nitrogen at room temperature.
7) Dissolve the residue in 100 μl pyridine. Add 100 μl *bis*-(trimethylsilyl)-trifluoroacetamide, cap tightly and heat at 120°C for 30 min.
8) Cool vials and evaporate to dryness under nitrogen at room temperature.
9) Dissolve the residue in 500 μl decane containing 5% *bis*-(trimethylsilyl)-trifluoroacetamide and transfer to an autosampler vial.

evacuated sample tubes with that from a range of carbonated standards prepared in the same way (Scrimgeour and Rennie, 1988).

2.17.7.1 ^{13}C Analysis of Protein-Bound Leucine

To prepare the muscle samples for determination of [1-^{13}C]leucine enrichment, the samples were retrieved from their storage in liquid nitrogen and

weighed (typically 75-350 mg wet weight). They were then pulverised in liquid nitrogen with a pre-cooled mortar and pestle with removal of any obvious pieces of connective tissue. An aliquot (approximately 20 mg in weight) of the finely ground powder was taken for estimation of free amino acid concentrations, extracted with ice-cold perchloric acid (PCA) (see 2.17.4) and the residue hydrolysed in 6 mol/l hydrochloric acid (Table 2.9 and Table 2.10). Leucine was separated by preparative gas chromatography (Smith *et al.*, 1988). The carboxyl carbon of the isolated leucine was liberated as carbon dioxide by ninhydrin and its ^{13}C enrichment was determined with the breath gas system, optimised for small quantities of carbon dioxide (Scrimgeour *et al.*, 1988).

2.17.7.2 Derivatization

Initially the trifluoroacetate isobutyl ester derivative of the amino acids was the adduct chosen (Roach and Gehrke, 1969; Smith *et al.*, 1988) for the preparative GC separation of protein amino acids. However later, the method was improved by the use of MTBSTFA as the derivatizing reagent (Biermann *et al.*, 1986; Schwenk *et al.*, 1984). This has two major advantages in that the derivatization is a simple, rapid one step procedure and the recovery of amino acid from the preparative gas chromatography is much greater (≈98% cf 40%). Briefly, 50-75 μl of MTBSTFA (Phase Separation Ltd, Clwyd, UK) together with an equal volume of pyridine were added to each sample then heated in an oven at 80-90°C for 90 min, or until the sample was completely dissolved. The derivatization was carried out immediately prior to the preparative chromatography as the sample is readily

Table 2.9 Determination of Isotope Ratio of Muscular Bound Leucine

This analysis was based on the method of Smith *et al.* (1988), where ground muscle protein is washed and acid-hydrolysed, leucine is separated by preparative gas chromatography of trifluoroacetyl isobutyl esters, and isotope quantification is by isotope ratio mass spectrometry of the carboxyl carbon after liberation as CO_2 with ninhydrin.

Method

Protein Extraction

1) A weighed sample of muscle (about 100-300 mg) is ground to a fine powder in liquid nitrogen with a pre-cooled mortar and pestle.
2) Extract free amino acids by adding 3 ml perchloric acid (0.5 mol/l), vortex and stand in ice for 30 min. Centrifuge for 20 min (3000 RPM, 4°C) and decant supernatant
3) To the residue add a further 3 ml perchloric acid (0.2 mol/l) vortex to mix and decant the supernatant. Repeat two times.
4) Add 3 ml NaOH (0.5 ml), mix and incubate at 37°C for 60 min in a water bath. Remove 100 μl, which can be used for a protein assay. Add 2 perchloric acid (1 mol/l) and centrifuge (3000 RPM, 20 min).
5) Suspend in 2 ml of perchloric acid (0.2 mol/l), vortex, centrifuge and decant supernatant.
6) Add 5 ml perchloric acid (1 mol/l) resuspend the pellet and heat at 70°C for 30 min in a water bath. Centrifuge (3000 RPM, 20 min) and decant supernatant.
7) Suspend the protein pellet in 3 ml HCL (mol/l) and transfer to a hydrolysis tube. Heat at 120°C overnight.

Ion-Exchange Chromatography

8) Transfer to a centrifuge tube and remove the solvent by rotary evaporation. Dissolve the residue in 50% glacial acetic acid.
9) Apply to a 2.5 ml column of ion-exchange resin (Dowex 50W-X8(H^+), 100-200 mesh, prewashed with 1 mol/l HCL). Rinse out the hydrolysis tube with 2x1 ml HCL (1 mol/1). Rinse the column with deionized distilled water (2x1 ml).
10) Elute the amino acids with 3 ml ammonia solution (4 mol/l). Collect the eluent into reaction vials, and blow-off the ammonia under nitrogen for about 30 min at room temperature (until pH is 7-9). Freeze with liquid nitrogen and lyophilize overnight.

Table 2.10 Determination of Isotope Ratio of Muscular Bound Leucine

Derivatization Procedure

11) Add an aliquot of dichloromethane (500 μl) and dry by evaporation under nitrogen at room temperature. Dissolve the residue in 400 μl 3 mol/l HCL-isobutanol and heat at 110°C for 30 min. Allow to cool and evaporate under nitrogen at room temperature.

12) Dissolve the residue in ethyl acetate (250 μl) and trifluoroacetic anhydride (100 μl) and react at 120-130 min for 30 min, releasing the pressure after 15 min. Allow to cool and evaporate off solvent under nitrogen at room temperature.

13) Dissolve in 100 μl of n-heptane and transfer to an autosampler vial. Reduce the volume to 100 μl under nitrogen at room temperature.

Preparative Gas Chromatography

14) An aliquot (50-100μl) of sample is injected into the injection port of a Pye 304 chromatography fitted with a wide-bore glass column (6 mm inside diameter x 4.6 m) packed with 2% EGA on 60-80 mesh Chromosorb W AW (Phase Sep, Queensferry, Clwyd, U.K.) and with a post column splitter (split ratio 99:1)

15) Argon was the carrier gas at a flow of 30 ml/min. The temperature of the injector was 200°C and the oven was programmed from 150 to 165°C at 2°C/min, then held at 165°C for 6 min before being increased to 220°C to drive off later eluting amino acid derivatives.

16) The leucine derivative is condensed in a demountable U-trap, cooled with liquid nitrogen. The leucine fraction is identified by comparison of retention times with these of leucine standards (about 11.5 min).

17) The leucine derivative is hydrolysed by adding 100 μl KOH (1 mol/l) and heating at 90°C for 45 min or leaving at room temperature overnight. The sample is transferred with 2 ml of buffer (pH 2) to a Vacutainer and is stored at 4°C overnight.

18) The vacutainer is cooled in ice before adding ninhydrin (25 mg) and a drop of antifoaming agent. After degassing and sealing, the sample is heated in a water bath at 90°C for 30 min to liberate the carboxyl-C as CO_2 for isotope ratio mass spectral analysis (Scrimgeour *et al.*, 1988).

hydrolysed by moisture.

2.17.8 Alkali-soluble protein, DNA and RNA estimation.

Alkali-soluble protein concentration of biopsy samples was determined by using the Lowry (Lowry *et al.*, 1951) method. Nucleic acids were extracted from the muscle samples (Schneider and Greco, 1971) and assayed by double-wavelength spectrophotometry (Tsanev and Markov, 1960).

2.17.9.1 Isolation and purification of plasma albumin

Plasma was stored at -80°C prior to the isolation of albumin. Total plasma protein was precipitated by the addition of ice cold 10% trichloroacetic acid (TCA, 4 ml) to plasma (1 ml) and centrifugation at 4°C for 20 min (1500 g), the supernatant was discarded. Albumin was first extracted into ethanol (5 ml), by vortex mixing then separated from the non-alcohol soluble protein by centrifugation at 4°C for 20 min (1500 g). The alcohol layer was decanted and evaporated under nitrogen at 70°C using the TurboVap® LV evaporator. This albumin containing and fraction was added to 10% SDS for subsequent polyacrylamide gel electrophoresis (PAGE) analysis (Laemmli *et al.*, 1970).

Contamination by free amino acids was minimized by redissolving the albumin in 0.3 N NaOH at 37°C for 30 min, then reprecipitating with ice cold 2M $HClO_4$ and discarding the supernatant. The protein pellet was then subjected to acid hydrolysis, purification of the acid hydrolysate by ion-exchange

chromatography, preparative gas chromatography and isotope ratio mass spectrometry of carboxyl-CO_2 as described for muscle.

2.17.9.2 Purity and Identification of Plasma Albumin

Plasma albumin was identified by polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli *et al.* (Laemmli *et al.*, 1970). Albumin was extracted from plasma into ethanol using the method described above. An aliquot of the ethanolic extract was removed and dried, then the protein solubilised in 10% sodium dodecyl sulphate (SDS) and an aliquot was removed and diluted with an equal volume of sample buffer. Separation was performed on a 10% acrylamide gel, using a 4% stacking gel run at 30 milliamps (mA, constant current) for approximately 1 h (Mini-Protean® II, Bio Rad, Hemel Hempstead, UK). The proteins were stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol, 10% glacial acetic acid overnight, then visualized by destaining with a 10% methanol, 70% acetic acid solution. A low molecular weight standard protein mix (Sigma, UK) and authentic human albumin (Immuno, Italy) were run in parallel in order to assess the molecular weight and verify the identity of protein in the alcohol soluble fraction. Purity was determined by the presence or absence of other protein bands; in several gels a low molecular weight protein band was observed. Semi-quantitation of the relative abundances of the albumin band and this unidentified low molecular weight protein was performed using a laser densitometer (2202 Ultroscan, LKB, Sweden) linked to a chart recorder. Measurement of the relative peak heights suggested that this band represented less

than 2% of the total purified. It was also possible that this protein fragment was a cleavage product from albumin generated during the isolation procedure.

2.17.10 Free Amino Acid Concentrations in Plasma and Muscle

Amino acid concentrations were determined in sulphosalicyclic acid extracts of plasma and perchloric acid (0.5 mol/l) extracts of muscle. Automated amino acid analyzers were used employing either a) ion-exchange chromatography with elution and separation of amino acids with strongly acidic lithium citrate buffers, norvaline as internal standard, post-column derivatisation with o-pthalaldehyde/-2-mercaptoethanol and fluorometric detection (LC5000, Biotronic GmbH, Munchen, F.D.R.); b) ion-exchange chromatography, lithium citrate buffers, no internal standard, post-column derivatization with ninhydrin and photometric detection (LC5000).

2.17.11 Plasma Hormone Concentrations

Plasma hormones were measured by radioimmunoassay. Insulin was determined with an enzyme-linked immunoabsorbent assay (Enzymun-Test®, Boehringer Mannheim, UK). The co-efficient of variation was 7%-9%. Glucagon was determined by a commercially available kit (Novo Nordisk, Denmark) and the co-efficient of between assay variation was 7%-12% over the assay range. Insulin-like growth factor 1 was determined with a double-antibody kit (Nicols Institute

Diagnostics, Essex, UK). The inter-assay co-efficient of variation was 8%-12%. C-peptide was measured with a commercially available kit (Novo Nordisk, Denmark); the co-efficient of between assay variation was 5%-8% over the assay range.

2.17.12 D-(-)-3-Hydroxybutyrate Concentration

The blood concentration of D-(-)-3-hydroxybutyrate was measured by an enzymic fluorometric continuous flow assay (Roche Diagnostics, Herts., UK) and the inter-assay co-efficient of variation was 2%-9%.

2.17.13 Blood Gas Analysis

The PO_2, oxygen saturation, pH and total bicarbonate concentration of arterial and venous blood was measured using a Corning 189 blood gas analyzer (Corning Medical, Medfield, Massachussetts, U.S.A.).

2.18 Statistical Analysis

In the text as well as in the tables and figures, mean values and standard errors are presented. Results from the two phases within studies were compared with the two-tailed Student's t-test for paired data, except where otherwise stated. In general, 95% confidence intervals have also been given throughout. The Student's t-test for unpaired data was used to compare different study groups, with

significance assigned at the 5% level i.e. $p < 0.05$. A stepwise regression analysis was performed to compare rates of protein synthesis with intramuscular free glutamine concentration. A microcomputer-based statistical package was used for the calculations (Minitab Statistical Software, Release 8.2, Minitab Inc., USA).

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CHAPTER 3

STIMULATION OF M. ANTERIOR TIBIALIS PROTEIN SYNTHESIS IN HEALTHY POSTABSORPTIVE MAN BY INFUSION OF THE DIPEPTIDE ALA-GLN

CONTENTS

TABLES

FIGURES

3.1 SUMMARY

In this study the acute effects of L-alanyl-L-glutamine (Ala-Gln) dipeptide infusion on protein synthesis in human anterior tibial muscle have been investigated. Fifteen healthy male post-absorptive subjects were studied: seven subjects received an infusion of Ala-Gln (11.67 mg/kg/h) and eight subjects received an isonitrogenous infusion of L-alanine (4.78 mg/kg/h) plus glycine (8.03 mg/kg/h) (Ala+Gly). Whole body and skeletal muscle protein synthesis were measured using a primed, constant infusion of [1-^{13}C]leucine with determination of plasma leucine-C flux and $^{13}CO_2$ production, and assay of [1-^{13}C]leucine incorporation into mixed anterior tibial muscle protein sampled by biopsy.

Arterialized venous blood glutamine concentration was higher during Ala-Gln than Ala+Gly infusion (640±40 (448, 948) *versus* 563±22 (506, 620) μmol/l, respectively, mean±SEM (95% confidence intervals), p=0.03). Intramuscular free glutamine concentration was also elevated after Ala-Gln infusion (78±9 (56, 100) *versus* 58±5 (46, 71)) μmol/g protein, p<0.02). Intramuscular free branched-chain amino acid concentrations were not significantly different (4.2±0.4 (3.0, 5.4) *versus* 3.3±0.3 (2.5, 4.1) μmol/g protein).

Muscle protein synthesis, calculated assuming plasma α-ketoisocaproate as precursor, was greater with Ala-Gln than with Ala+Gly infusion (0.051±0.003 (0.044, 0.058) *versus* 0.042±0.001 (0.039, 0.045) %/h, p<0.03). Whole body leucine flux and oxidation values, although similar in the two groups (104±4 (94, 115) *versus* 115±4 (105, 125), and 15±1.5 (11, 20) *versus* 17±1.4 (13, 21) μmol/kg/h, respectively), tended to be lower with Ala-Gln infusion. Non-oxidative leucine disposal, i.e. whole body protein synthesis, also did not differ significantly (90±3

(81, 98) *versus* 98±4 (87, 109) μmol/kg/h), between the two groups.

These results suggest that in healthy man increased availability of glutamine alone, provided as the dipeptide Ala-Gln, may acutely stimulate muscle protein synthesis in the post-absorptive state.

3.2 Introduction

It is well recognised that skeletal muscle plays a key role in the metabolic response to injury and sepsis and in the negative nitrogen balance often associated with these conditions. Characteristically, skeletal muscle wasting occurs resulting in efflux of amino acids which are used as substrates for gluconeogenesis, wound healing and visceral protein synthesis (Rennie, 1985). In particular, loss of muscle-derived amino acids to the viscera includes the 'non-essential' amino acid glutamine, the most abundant free amino acid in human skeletal muscle (Fürst, 1983) and a significant diminution occurs (up to 60%) in the intramuscular free glutamine pool (Vinnars *et al.*, 1975; Askanazi *et al.*, 1980a; Askanazi *et al.*, 1980b; Roth *et al.*, 1982; Fürst, 1984). In several recent studies the effects of glutamine on the rates of protein synthesis and breakdown in skeletal muscle have been investigated, both *in vitro* and *in vivo*. Thus glutamine has been shown to inhibit protein breakdown in cultured rat and chick skeletal muscle (Smith, 1985; Wu and Thompson, 1990). Also, Wu and Thompson demonstrated an increase in protein synthesis in chick skeletal muscle incubated with 2-15 mM glutamine *in vitro* (Wu and Thompson, 1990) and in perfused rat hindlimb there is an anabolic relationship between glutamine availability and muscle protein turnover (MacLennan *et al.*,

1987. Fasted, injured and endotoxin-treated rats show a positive relationship between intramuscular glutamine concentration and the rates of muscle protein synthesis *in vivo* (Jepson *et al.*, 1988). The evidence that maintenance of the intracellular glutamine pool may help conserve muscle protein is strengthened by the findings of Kapadia and co-workers who achieved a reduction in efflux of amino acids in dog hindlimb and sustained intramuscular glutamine levels following surgical trauma by infusing an amino acid solution containing glutamine (Kapadia *et al.*, 1985). Thus it would seem desirable, in the clinical context, to include glutamine in parenteral nutrition formulations. Hammarqvist and colleagues have demonstrated that such a solution administered to patients undergoing elective abdominal surgery reduces the fall in intramuscular free glutamine, counteracts the fall in muscle polyribosome concentration (used as an index of muscle protein synthesis) and improves nitrogen balance (Hammarqvist *et al.*, 1989).

Practical difficulties may arise however in administering glutamine-containing parenteral nutrition regimes as glutamine is unstable when stored in solution (Stehle *et al.*, 1984), forming the possibly toxic substance pyroglutamic acid (Fürst *et al.*, 1987). Provision of glutamine in an alternative, stable form as the dipeptide L-alanyl-L-glutamine (Ala-Gln) (Stehle *et al.*, 1984; Fürst *et al.*, 1987; Albers *et al.*, 1988; Albers *et al.*, 1989) increases glutamine availability, reduces post-operative muscle loss of alanine and glutamine (Roth *et al.*, 1988) and whole-body N-excretion (Stehle *et al.*, 1989), although no data are currently available to explain the mechanisms of this action in human subjects. We have therefore begun by investigating the possible acute effects of Ala-Gln infusion on muscle protein synthesis and whole-body protein turnover in healthy male postabsorptive subjects using standard tracer techniques (Chapter 2).

3.3 Methods

3.3.1 Subjects

Fifteen healthy male subjects (age 24-52 y, weight 63.8-84.5 kg) were studied in the Department of Surgery, Ninewells Hospital and Medical School, Dundee University. Each subject gave written consent after a full explanation of the study. The protocol for the study was approved according to the Declaration of Helsinki guidelines by the Dundee General Hospitals Committee on Medical Ethics of the Tayside Health Board.

3.3.2 Study Design

Subjects (Table 3.1) were randomised to receive either Ala-Gln infusion (n=7, age 33.1±8 y, 95% confidence intervals (25, 42); weight 72.6±7 (66, 79) kg; body-mass index 25.1±2.3 (23, 27) $kg.m^{-2}$; fat-free mass 54.8±6 (48, 62) kg, as estimated from calliper-measured skin-fold thicknesses (Durnin and Womersley, 1973) *or* isonitrogenous Ala *plus* Gly (n=8, age 33.3±8 (26, 41) y ; weight 72.5±8 (66, 80) kg ; body-mass index 23.5±1.2 (22, 25) $kg.m^{-2}$; fat-free mass 57.2±5 (53, 62) kg) (Protocol 1, Chapter 2). After an overnight 15 h fast, subjects were studied in the supine position in a thermoneutral environment (25-27°C). No food or water was given to the subjects until the study was complete. Whole-body tracer leucine-C kinetics and the incorporation of [1-^{13}C]leucine into M. tibialis anterior were investigated over an 8 h period in each group.

Table 3.1 Age, morphology and pre-study nutritional state in the two groups of subjects. CI = 95% confidence intervals.

	Ala+Gly			Ala-Gln		
	Mean ± SEM	Range	CI	Mean ± SEM	Range	CI
Age (y)	33.3 ± 3.2	24-52	(25.7, 40.8)	33.1 ± 3.4	24-49	(24.8, 41.5)
Wt (kg)	72.5 ± 3.0	63.8-84	(51.7, 73.7)	65.1 ± 5.1	66.0-84.5	(52.2, 78)
Body fat (%)	21.0 ± 1.3	16.2-26.5	(17.9, 24)	21.9 ± 1.1	20.0-27.0	(19.2, 24.6)
Fat (kg)	15.4 ± 1.4	10.8-20.5	(10, 16.4)	14.3 ± 1.4	10.0-24.5	(10.8, 17.8)
FFM (%)	78.1 ± 1.1	73.5-83.0	(76, 82.1)	78.1 ± 1.1	72.9-80.0	(75.4, 80.8)
FFM (kg)	57.2 ± 1.9	50.3-64.3	(40.9, 58.1)	50.7 ± 3.9	49.5-66.8	(40.8, 60.7)
MAC (cm)	29.5 ± 0.8	28-33	(24.6, 29.1)	26 ± 0.8	26.5-31.0	(23.8, 28.1)
BMI	23.5 ± 0.5	22.2-26.2	(20.2, 25.6)	23.5 ± 1.2	21.0-28.7	(20.8, 26.3)

Wt = Weight
FFM = Fat free mass
MAC = Mid arm circumference
BMI = Body mass index

3.3.3 Materials

Solutions of L-[1-^{13}C]leucine and sodium ^{13}C-bicarbonate (both 99 Atoms %, Tracer Technologies Inc., Newton, MA, U.S.A.) were prepared under aseptic conditions in sterile, pyrogen-free 0.9% (150 mmol/l) sodium chloride solution (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.). The solutions were sterilized by filtration through 0.22μm filters (Millipore SA, Molsheim, France). Solutions were similarly prepared of the dipeptide Ala-Gln (Kabi-Pfrimmer, Erlangen, F.D.R.) and a mixture of the unlabelled amino acids L-alanine and glycine (Sigma Laboratories, U.K.).

3.3.4 Study Details

Arterialized venous blood ('arterial' blood) sampling was carried out via a 1.7 mm outer diameter cannula (Venflon, Viggo AB, Helsingborg, Sweden), placed retrogradely in a dorsal hand vein and maintained patent by slow infusion of 150 mmol/l NaCl solution. Before each sampling the hand was warmed for 10 min by insertion in a thermostatic chamber heated to 65°C (Abumrad *et al.*, 1981). A second cannula was placed antegradely in an antecubital vein of the ipsilateral arm for tracer amino acid and dipeptide infusions. The third cannula was inserted retrogradely in an antecubital vein of the contralateral forearm for venous blood sampling. A paediatric sphygmomanometer wrist cuff was applied at the wrist on this side, with the cuff being inflated to 300 mmHg 2 minutes prior to blood sampling in order to

avoid admixture of the predominantly cutaneous venous drainage of the hand with the venous drainage from the muscle bed of the forearm (Jackson *et al.*, 1987). Priming doses of L-[1-^{13}C]leucine (1 mg/kg) and $NaH^{13}CO_3$ (0.20 mg/kg) were administered (Matthews *et al.*, 1980; Nair *et al.*, 1988b) followed by a constant infusion of tracer L-[1-^{13}C]leucine (1 mg/kg/h) with Ala and Gly (4.78 and 8.03 mg/kg/h respectively, control group) or L-[1-^{13}C]leucine with Ala-Gln (11.67 mg/kg/h, study group) maintained and delivered over 8 h with an IMED 928 volumetric infusion pump (IMED Ltd, Abington, U.K.). Blood samples were taken before the start of the tracer infusion and at intervals throughout the study (30, 90, 150, 240, 330, 420, 480 min) for blood gas analysis (P_{CO_2}, P_{O_2}, SaO_2% - Corning 178 Blood Gas Analyzer, CIBA Corning Diagnostics, Essex, U.K.), amino acid concentrations and for determination of ^{13}C enrichment of (plasma) α-ketoisocaproate (α-KIC). In order to obtain base-line values for [1-^{13}C]leucine enrichment of body protein, against which to compare the incorporation of tracer [1-^{13}C]leucine into muscle protein, a 5 ml blood sample was collected prior to the tracer infusion and mixed immediately with 5 ml 2M perchloric acid for precipitation, and later isolation and analysis, of whole-blood protein (Heys *et al.*, 1990). Expired air was collected into evacuated glass tubes (Vacutainer, Becton Dickinson, Rutherford, U.S.A.) for short-term storage prior to ^{13}C analysis of CO_2. CO_2 production rate was determined over the 8 h period by two separate 10 min collections of expired breath into a 100 litre Douglas bag, the volume being determined by a Harvard dry gas meter. Samples of the breath (200 ml) were subsequently analyzed for CO_2 with an infra-red absorption CO_2 gas analyzer (Analytical Development Co., Ltd., U.K.). Muscle biopsy was carried out after 8 h of infusion. After infiltration of the skin and

subcutaneous tissues down to the deep fascia with 5 ml 1% lignocaine, a 10 mm skin incision was made over the tibialis anterior at mid-leg level and using 6.5 mm Tilley-Henckel ethmoid punches (S. Murray and Co., Sheffield, U.K.) a 150-200 mg sample of muscle tissue was obtained (Dietrichson *et al.*, 1987) and stored in liquid nitrogen until analysis.

3.3.5 Analysis of Samples

The laboratory methods of analysis of samples used here have been detailed previously in Chapter 2, section 2.17.

3.3.6 Calculations

Muscle protein synthetic rate was calculated from L-[1-^{13}C]leucine incorporation into the protein-bound leucine pool. Calculations were made assuming that labelling of the precursor pool of leucyl-*t*RNA was approximated by venous plasma α-KIC (Rennie *et al.*, 1982b) and assuming that basal ^{13}C enrichment of leucine in whole blood proteins reflected the basal ^{13}C enrichment in protein-bound leucine in muscle (Heys *et al.*, 1990). The components of plasma leucine turnover were calculated from the mean arterial α-KIC enrichment from 330 to 480 min. Leucine oxidation was calculated from whole-body $^{13}CO_2$ excretion and the arterial plasma α-KIC enrichment. Intramuscular free amino acid concentrations were expressed per g of alkali-soluble muscle protein.

3.3.7 Statistical Analysis

Intergroup differences in results were analyzed with the Student's *t*-test for non-paired data (two-tailed). When comparing intra-group differences as for example in results of basal and plateau plasma amino acid concentrations, Student's *t*-test for paired data was used (two-tailed, except where otherwise stated). Both in the tables and in the text, 95% confidence intervals around the mean are presented.

3.4 Results

3.4.1 Plasma Free Amino Acid Concentrations

The basal arterial plasma Gln concentration in the control group was 555±22 (498, 611) μmol/l (mean±SEM (95% CI)) and 563±22 (506, 620) μmol/l during the period 90 to 480 min, thus showing little change during the study period (Table 3.2 and Figure 3.1). In the Ala-Gln group the basal glutamine concentration was 566±36 (476, 651) μmol/l, rising (p=0.03) to a plateau concentration of 640±43 (534, 746) μmol/l, between 90 to 480 min. There were no significant differences in either the basal or the peri-infusion concentrations of alanine between the groups (258±17 (216,300) and 291±34 (206, 376) μmol/l, control, *versus* 243±29 (166, 320) and 274±24 (210, 337) μmol/l, Ala-Gln). The plasma glycine concentration rose sharply (249±10 (224, 274) *versus* 443±14 (408, 479) μmol/l, p<0.001) in the control group during infusion of Ala+Gly. The

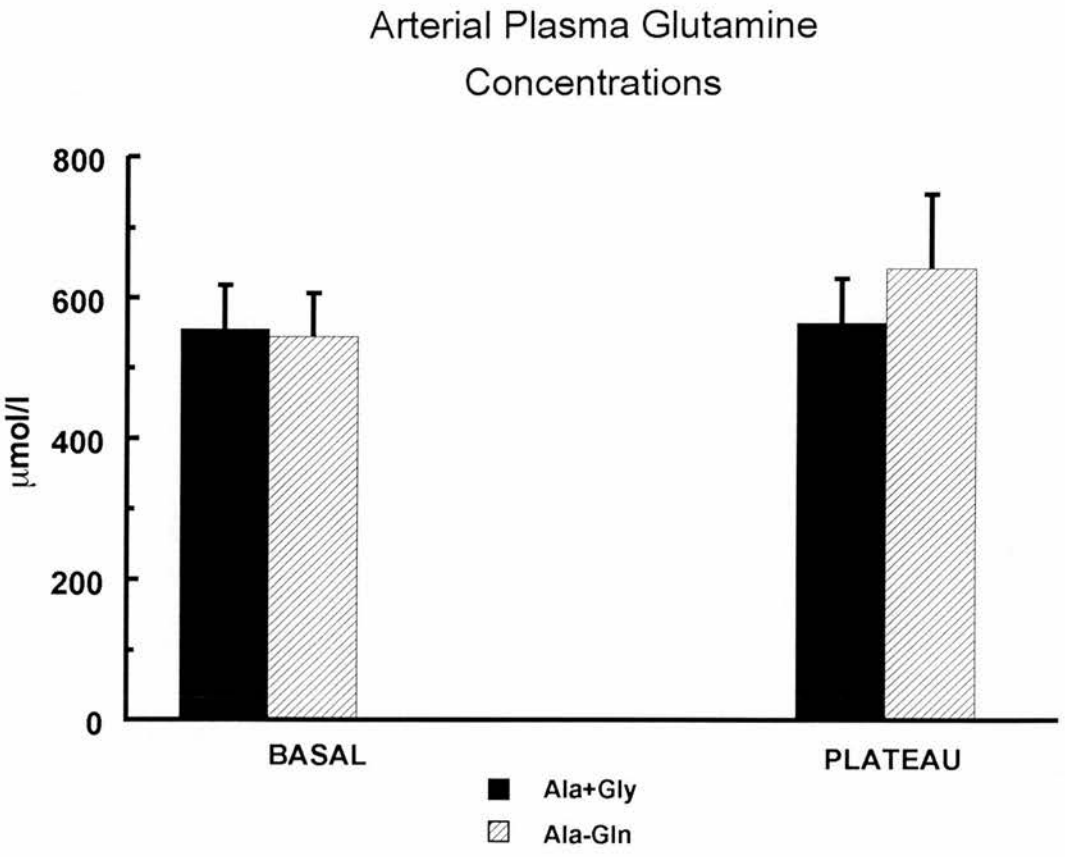

Figure 3.1 Basal and plateau (90-480 min) free glutamine concentrations in arterial plasma, in the control group receiving Ala+Gly (solid bar) and study group receiving Ala-Gln (hatched bar).

Table 3.2 Free amino acid concentrations in plasma. BCAA, total branched chain amino acids. The results are means±SEM. CI = 95% confidence intervals.

	Amino acid conc. (μmol/l)							
	Ala+Gly				Ala-Gln			
	Basal	C.I.	90-480 min	C.I.	Basal	C.I.	90-480 min	C.I.
ALA	258±17	(216, 300)	291±34	(206, 376)	243±29	(166, 320)	274±24	(210, 337)
GLU	66±3.9	(55, 76)	49±2.5	(43, 56)	72±8.7	(49, 95)	57±8	(34, 79)
GLN	555±22	(498, 611)	563±22	(506, 620)	544±23	(476, 651)	640±40	(534, 746)
GLY	249±10	(224, 274)	443±14	(408, 479)	203±7	(185, 220)	179±6	(162, 196)
3-MH	4.8±0.6	(3, 6)	4.1±0.4	(3, 5)	5.3±0.6	(3.8, 6.7)	4.1±0.4	(3.1, 5.2)
ISO	64±3.9	(54, 74)	74±20	(22, 125)	67±5	(53, 82)	57±2.6	(49, 64)
LEU	139±9.2	(116, 163)	174±12	(143, 205)	138±9	(128, 160)	178±5.2	(164, 191)
PHE	57±3.9	(47, 66)	59±2.5	(53, 65)	53±4	(50, 57)	59±0.8	(57, 61)
TYR	50±2.5	(43, 56)	40±2	(34, 45)	60±12	(37, 59)	44±3	(36, 51)
VAL	227±15	(191, 264)	212±14	(177, 246)	231±19	(177, 258)	206±4	(195, 217)
BCAA	431±27	(363, 499)	459±38	(363, 555)	429±22	(371, 487)	440±11	(413, 468)

Table 3.3 Intramuscular free glutamine concentrations. CI = 95% confidence intervals.

Ala+Gly		Ala-Gln	
Subject	Gln conc. (μmol/g protein)	Subject	Gln conc. (μmol/g protein)
1	53.6	9	61.3
2	36.7	10	104.9
3	54.5	11	67.4
4	43.5	12	116.4
5	68.2	13	87.0
6	64.3	14	50.0
7	84.5	15	58.4
8	59.3		
Mean$\pm$SEM	58.1$\pm$5		77.9$\pm$9
CI	(45.7, 70.5)		(55.6, 100.2)

plasma glycine concentration fell (203$\pm$7 (185, 220) *versus* 179$\pm$6 (162, 196) μmol/l, $p<0.02$) in the Ala-Gln group. Other amino acid concentrations (L-leucine, L-phenylalanine, L-tyrosine and L-glutamic acid) also changed from their basal values in both groups, although there was no significant difference in the values between the groups.

3.4.2 Intramuscular Free Amino Acid Concentrations.

The intramuscular free Gln concentration (Tables 3.3, 3.4 and Figure 3.2)

Table 3.4 Intramuscular free amino acid concentrations. BCAA, total branched chain amino acids. The results are means±SEM. CI = 95% confidence intervals.

	Amino acid concn. (μmol/g protein)			
	Ala+Gly	CI	Ala-Gln	CI
ALA	9.3±0.5	(8.1, 10.5)	12.4±3.1	(3.8, 21)
ARG	1.6±0.1	(1.4, 1.8)	2.5±0.6	(0.7, 4.3)
ASP	4.7±0.7	(2.9, 6.5)	3.6±1.1	(0.5, 6.7)
GLU	21.9±2.4	(15.8, 28.1)	24±2.7	(16.3, 31.7)
GLN	58.1±5	(45.7, 70.5)	78±9	(55.6, 100.2)
GLY	10.0±1.0	(7.5, 12.6)	6.5±0.7	(4.4, 8.6)
HIS	2.0±0.1	(1.6, 2.3)	2.3±0.5	(1.0, 3.6)
ISO	0.3±0.03	(0.2, 0.4)	0.5±0.04	(0.4, 0.6)
LEU	1.3±0.1	(1.0, 1.6)	1.7±0.2	(1.3, 2.2)
LYS	12.4±6.6	(-4,4, 29.2)	40.2±26.4	(-34.1, 114.5)
PHE	0.5±0.1	(0.4, 0.7)	0.7±0.1	(0.3, 1.1)
SER	3.2±0.2	(2.7, 3.7)	3.4±0.6	(1.8, 4.9)
TAU	95.2±12	(65.7, 124.7)	119.7±14	(80.4, 158.9)
THR	2.6±0.1	(2.3, 3.0)	2.2 ±0.6	(0.5, 3.8)
TRY	36.5±14	(-2.7, 75.7)	27.5±12	(-6.4, 61.4)
TYR	0.30±0.04	(0.2, 0.5)	0.4±0.1	(0.2, 0.7)
VAL	1.7±0.2	(1.2, 2.1)	2.0±0.2	(1.3, 2.7)
BCAA	3.3±0.3	(2.5, 4.1)	4.2±0.4	(3.0, 5.4)

was greater in the Ala-Gln group (78±9 (56, 100) *versus* 58±5 (49, 71) μmol/g protein, $p<0.02$). Isoleucine and leucine concentrations (Figure 3.3) were also higher in this group (0.5±0.04 (0.4, 0.6) *versus* 0.3±0.03 (0.2, 0.4) μmol/g protein, $p<0.01$, and 1.7±0.2 (1.3, 2.2) *versus* 1.3±0.1 (1.0, 1.6) μmol/g protein, $p<0.05$, respectively).

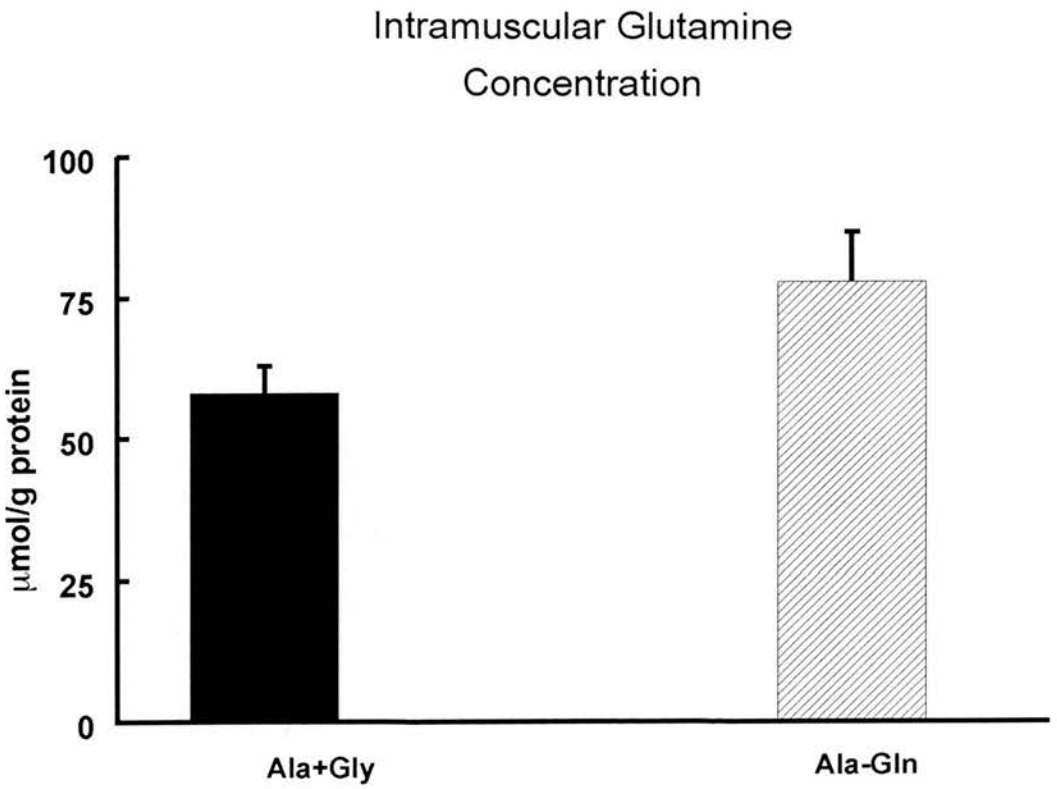

Figure 3.2 Intramuscular free glutamine concentrations, in the group receiving Ala+Gly (solid bar) and the group receiving Ala-Gln (hatched bar).

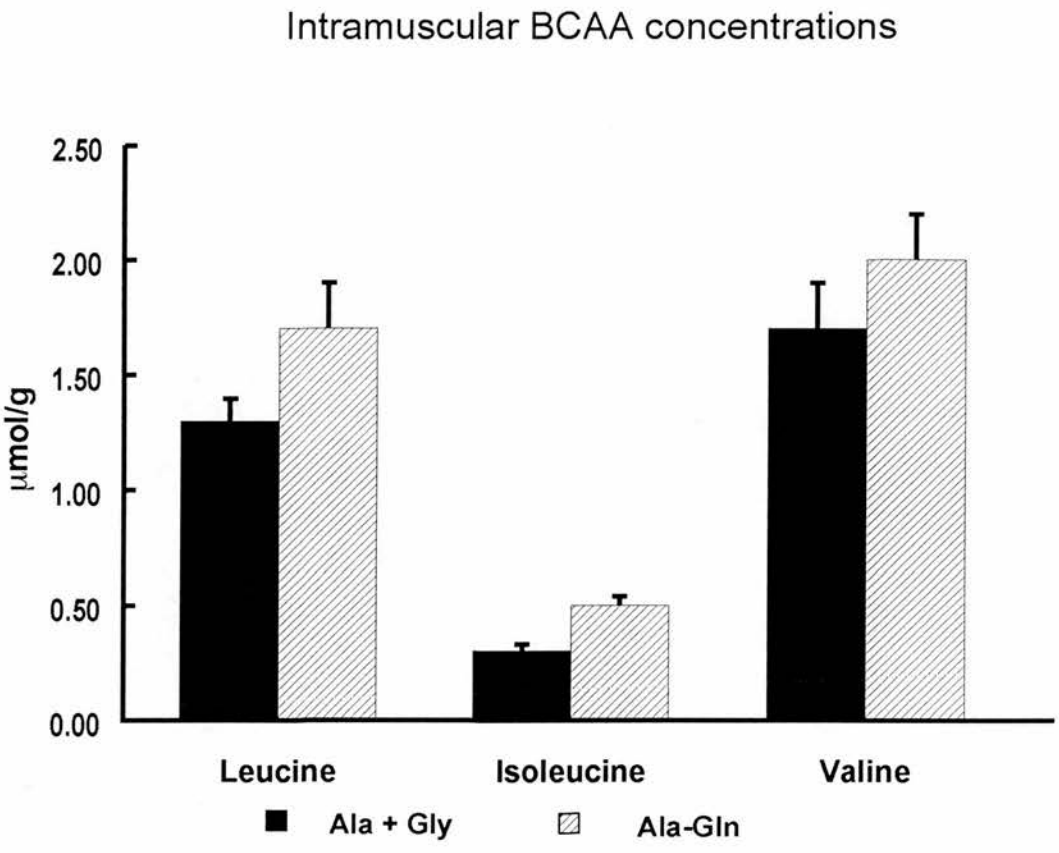

Figure 3.3 Intramuscular concentrations of the free branched chain amino acids leucine, isoleucine and valine in the Ala+Gly group (solid bar) and Ala-Gln group (hatched bar).

3.4.3 Plasma Concentrations and ^{13}C Enrichment of Leucine and α-KIC

Plateau values were attained for the enrichment of 'arterial' plasma leucine and α-ketoisocaproate during the periods 90-480 min of each study (Figures 3.4 and 3.5). There were no significant differences in enrichment of leucine and α-ketoisocaproate between the two groups in either *arterial* (plasma leucine APE: 6.9±0.4 (6.05, 7.82), Ala+Gly *versus* 7.5±0.1 (7.13, 7.81), Ala-Gln; α-KIC APE: 5.5±0.3 (4.8, 6.2), Ala+Gly *versus* 6.0±0.3, (5.2, 6.7) Ala-Gln) or *venous* (leucine APE: 5.8±0.4 (4.9, 6.7), Ala+Gly *versus* 6.0±0.2 (5.5, 6.5), Ala-Gln; α-KIC APE: 4.8±0.3 (4.0, 5.6), Ala+Gly *versus* 5.2±0.3 (4.4, 6.0), Ala-Gln) plasma. The concentrations of 'arterial' plasma leucine and α-ketoisocaproate were stable during the periods 90-480 min of each study (Figures 3.6 and 3.7) and there were no significant differences in the concentrations of either, during this period, between the two groups.

3.4.4 Fractional Muscle Protein Synthetic Rate

The muscle fractional mixed protein synthetic rate, calculated assuming plasma α-ketoisocaproate (Table 3.5 and Figure 3.8) as precursor, was greater with Ala-Gln infusion than with Ala+Gly infusion (0.051±0.003 (0.044, 0.058) *versus* 0.042±0.001 (0.039, 0.045)) %/h, $p<0.03$).

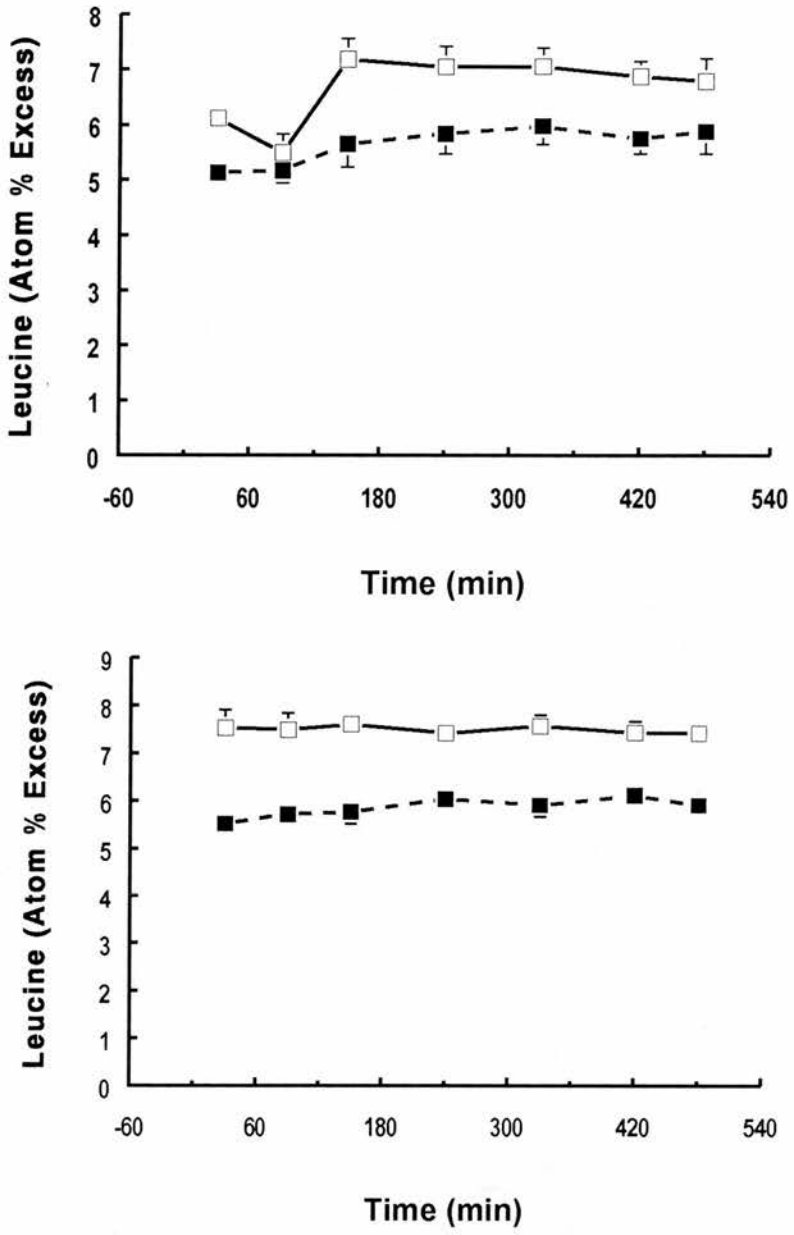

Figure 3.4 Plasma arterial (□) and venous (■) enrichment of free leucine in the Ala+Gly group (top graph) and Ala-Gln group (bottom graph).

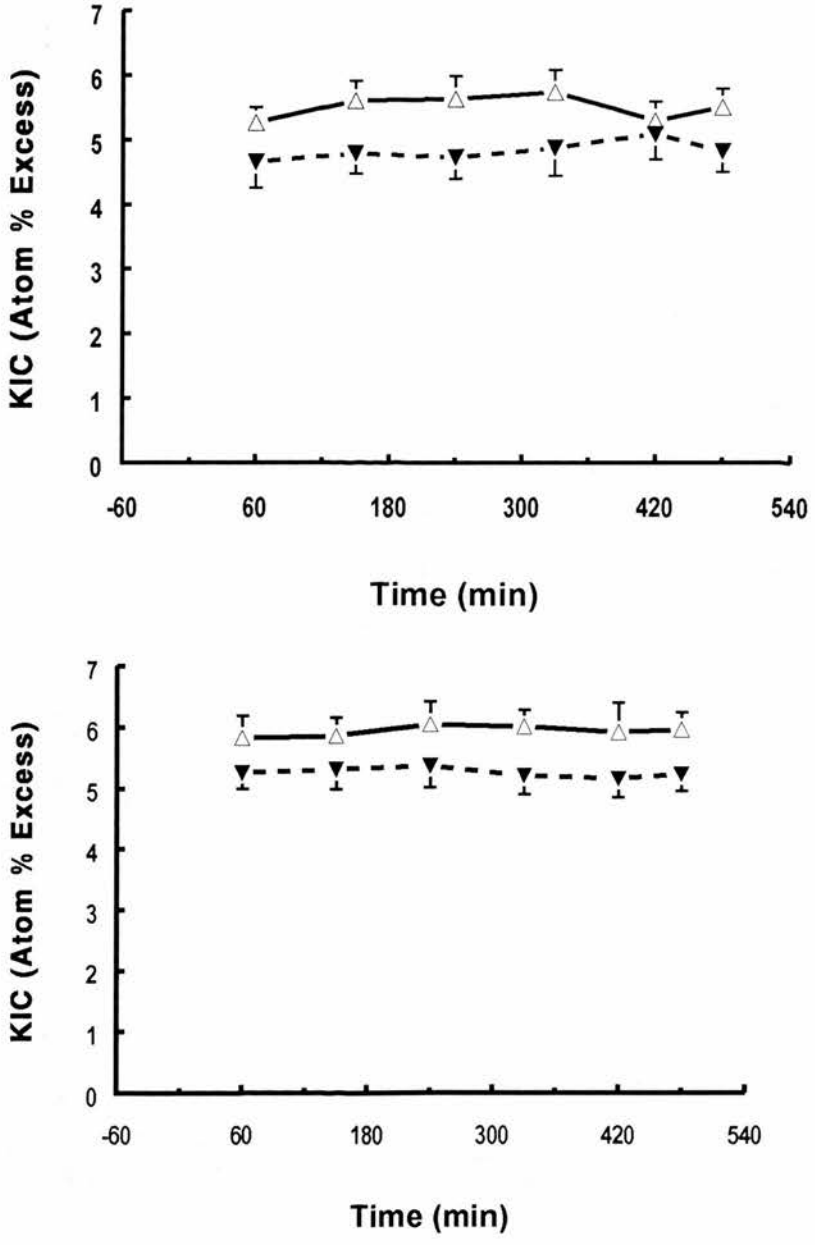

Figure 3.5 Plasma arterial (Δ) and venous (▾) enrichment of α-ketoisocaproate in the Ala+Gly group (top graph) and Ala-Gln group (bottom graph).

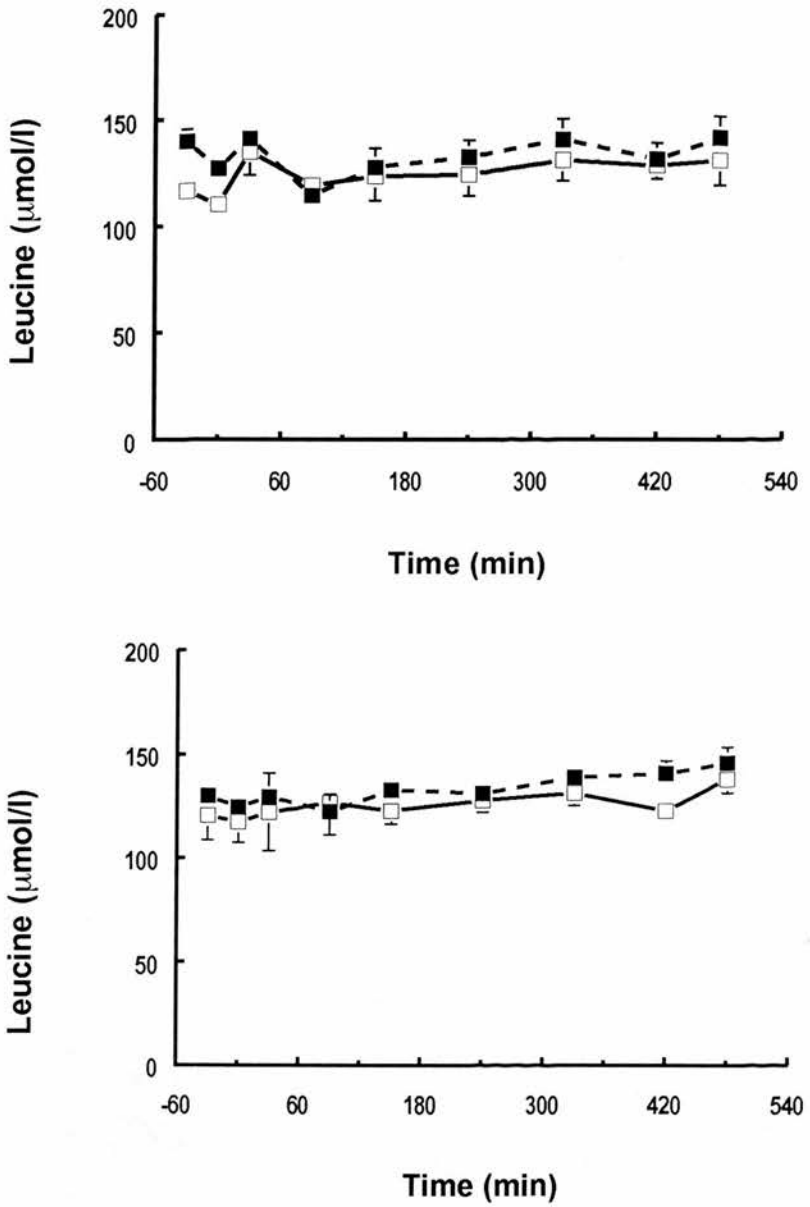

Figure 3.6 Plasma arterial (□) and venous (■) concentrations of free leucine in the Ala+Gly group (top graph) and Ala-Gln group (bottom graph).

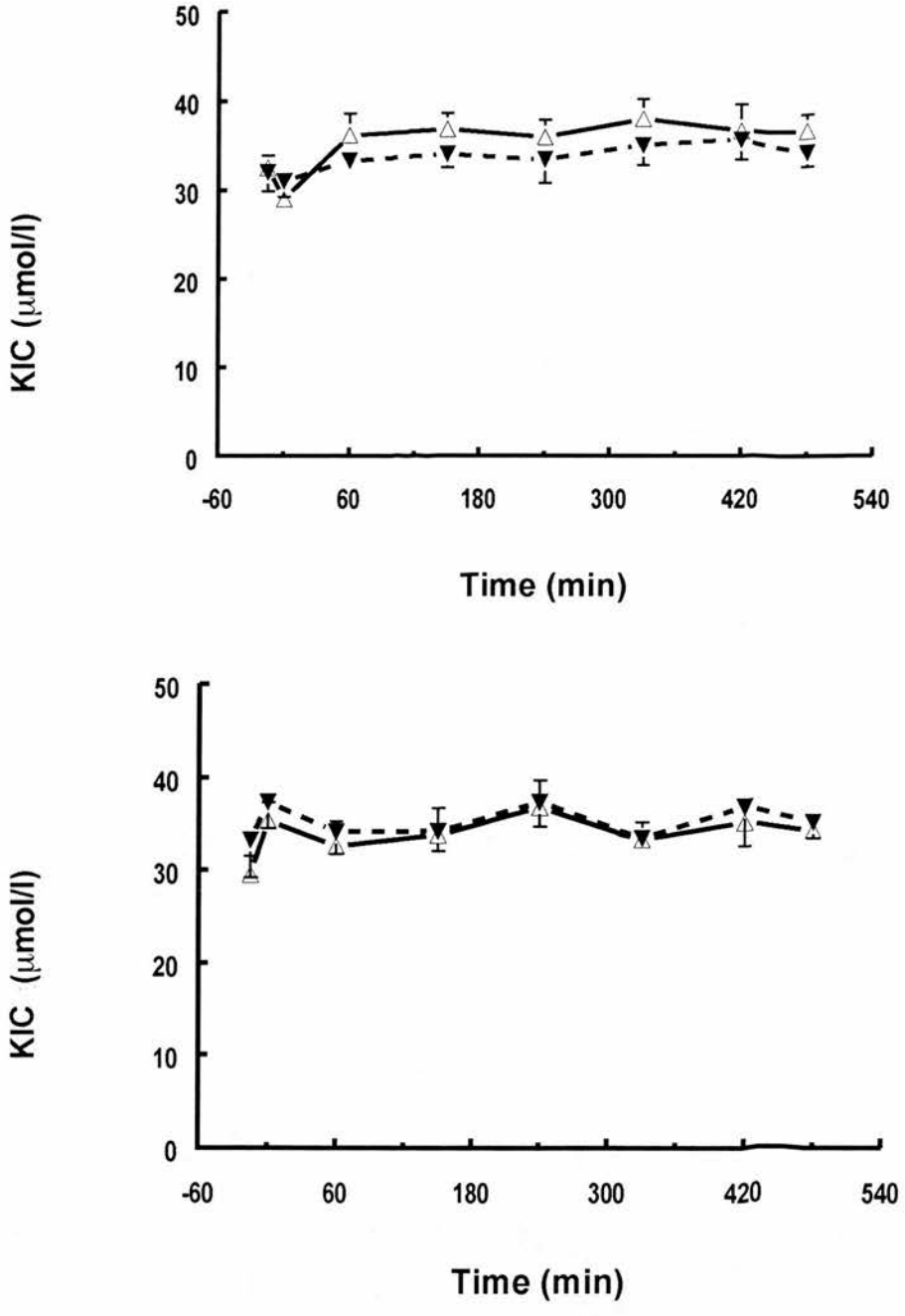

Figure 3.7 Plasma arterial (Δ) and venous (▾) concentrations of α-ketoisocaproate in the Ala+Gly group (top graph) and Ala-Gln group (bottom graph).

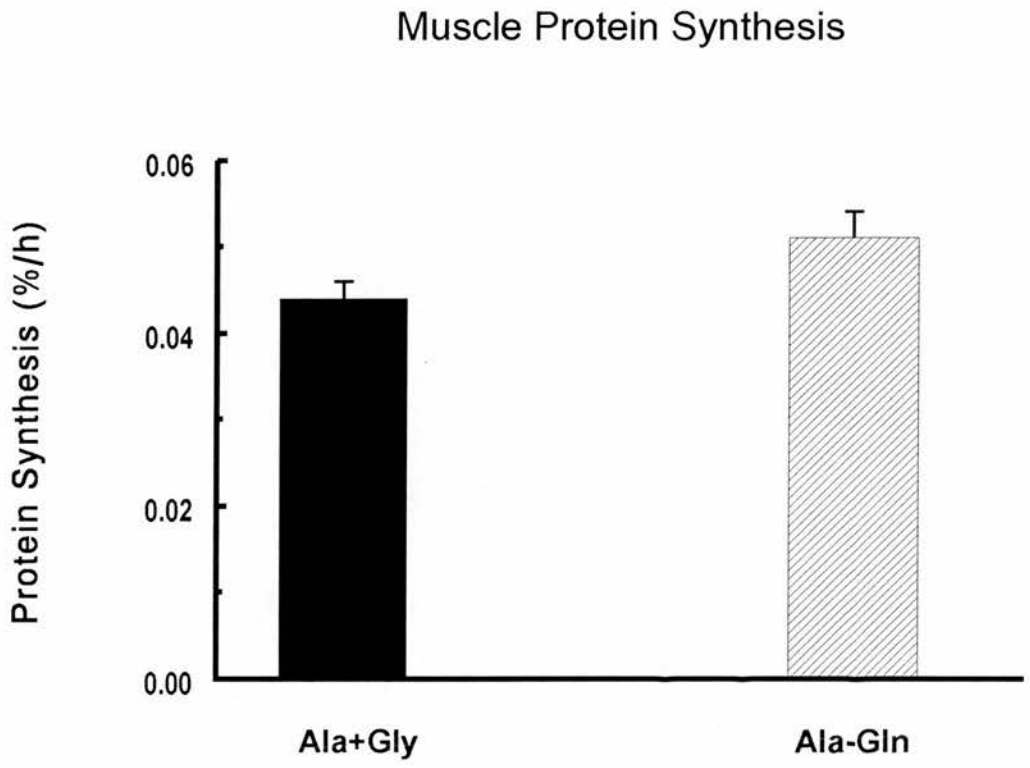

Figure 3.8 Skeletal muscle fractional mixed protein synthesis, calculated using the (surrogate) precursor pool of plasma α-ketoisocaproate, in the group receiving Ala+Gly (filled bar) and the group receiving Ala-Gln (hatched bar).

Table 3.5 Fractional protein synthetic rate (ks) in M. tibialis anterior. Calculated on the basis of α-KIC as precursor. CI = 95% confidence intervals.

Ala+Gly		Ala-Gln	
Subject	Ks (%h)	Subject	Ks (%h)
1	0.040	9	0.059
2	0.038	10	0.062
3	0.044	11	0.044
4	0.046	12	0.044
5	0.038	13	0.043
6	0.043	14	0.055
7	0.046	15	0.051
8	0.042		
Mean±SEM	0.042±0.001		0.051±0.003
CI	(0.039, 0.045)		(0.044, 0.058)

3.4.5 Protein/DNA, RNA/protein and RNA/DNA

There were no overall significant differences in the muscle samples from the Ala-Gln and Ala+Gly groups in protein/DNA (525±30 (450, 599) *versus* 544±21 (493,595) μg/μg) (Table 3.6), RNA/protein (2.9±0.17 (2.5, 3.3) *versus* 2.6±0.15 (2.3,3.0) μg/mg) or RNA/DNA (1.50±0.11 (1.2, 1.8) *versus* 1.43±0.09 (1.2, 1.6) μg/μg).

3.4.6 Whole-body Leucine Kinetics

Whole-body leucine kinetics (Table 3.7) showed no significant difference at

Table 3.6 Intramuscular protein/DNA, RNA/protein and RNA/DNA ratios. Results are means±SEM. CI = 95% confidence intervals.

	Ala+Gly	CI	Ala-Gln	CI
Protein/DNA (μg/μg)	544.2±21	(493, 595)	524.8±30	(450, 599)
RNA/protein (μg/mg)	2.64±0.15	(2.3, 3.0)	2.87±0.17	(2.5, 3.3)
RNA/DNA (μg/mg)	1.43±0.09	(1.2, 1.6)	1.50±0.11	(1.2, 1.8)

the 95% level between the Ala-Gln and Ala+Gly groups. However whole-body leucine flux and whole-body protein breakdown tended to be lower with Ala-Gln (both 104±4 (94, 115) μmol/kg/h) than with Ala+Gly (115±11 (105, 125) μmol/kg/h). Similarly, leucine oxidation (15±1.5 (11, 20) *versus* 17±1.4 (13, 21) μmol/kg/h) and whole-body protein synthesis (90±3 (81, 98) *versus* 98±4 (87, 109) μmol/kg/h) were a little lower, at 12 % and 8 % respectively, with Ala-Gln infusion.

3.5 Discussion

This study provides, for the first time, direct evidence for a small but significant acute stimulatory effect on skeletal muscle protein synthesis in man by glutamine administered parenterally as the dipeptide Ala-Gln. The infusion rate

Table 3.7 Whole-body leucine kinetics. Results are means±SEM. CI = 95% confidence intervals.

	Rate (μmol/kg/h)			
	Ala+Gly	CI	Ala-Gln	CI
Flux	115±4	(104.9, 124.9)	104±4	(94.2, 114.7)
Protein breakdown	115±4	(104.9, 124.9)	104±4	(94.2, 114.7)
Oxidation	17 ±1.4	(13.3, 21.0)	15±1.5	(10.8, 19.5)
Protein synthesis	98 ±4	(86.9, 108.6)	90±3	(80.5, 98.1)
Net protein balance	-17±1.4	(13.3, 21.0)	-15±1.5	(10.8, 19.5)

of Ala-Gln chosen for this study (11.67 mg/kg/h) was the highest that had previously been shown to be safe and effective in raising plasma glutamine concentrations in man (Stehle *et al.*, 1989). In this study we achieved greater plasma and intramuscular concentrations of free glutamine in the dipeptide treated group (Tables 3.2 and 3.3). With infusion of equivalent amounts of alanine in the free and dipeptide form (as Ala-Gln), there were no differences in its mean (free) intramuscular concentration between the respective groups. Infusion of glycine resulted in a significantly greater intramuscular concentration in the control group. We have no evidence that muscle protein synthesis was adversely affected by the greater intramuscular glycine as the synthesis rates are similar to previously published basal values for M. tibialis anterior in postabsorptive subjects from our laboratory (Bennet *et al.*, 1989). This finding is consonant with that of Jepson *et al* 1988) who found no relationship existed between the intramuscular glycine concentration and muscle protein synthesis in rats. Intramuscular concentrations

of the branched-chain amino acids leucine and isoleucine were significantly raised (by 32%, $p<0.05$ and by 50%, $p<0.01$, respectively) in the Ala-Gln group although there was no change in the concentration of valine. These changes differ qualitatively from those reported by Wu (Wu and Thompson, 1990) in chick extensor digitorum communis muscle *in vitro* in which concentrations of all three amino acids decreased with increased glutamine concentration. The discrepancy may in part be species related, or may be explained by the very different study conditions involved.

In investigating the effect of intervention on a biological variable (e.g. protein synthesis) in man it would seem probable that, unlike equivalent studies in laboratory animals of similar genetic types, the results may demonstrate a greater heterogeneity of response. This is evident in the present study (although the subjects were well-matched) in which there was a considerable variation in response to Ala-Gln and Ala+Gly infusions in the plasma and intramuscular free amino acid concentrations (see Tables 3.2 and 3.4) and in the muscle protein synthetic rates (Table 3.5). It is also possible that analytical factors contributed a little to the variability. Nevertheless, in this relatively small randomized study the differences in the mean values of intramuscular free glutamine concentration and muscle protein synthesis rates between the groups are significant. Additionally, there appears to be a trend towards a positive association between increased muscle glutamine concentration and increased muscle protein synthesis, although in view of the small numbers of subjects in each group the power of the study was insufficient to demonstrate any statistically significant correlation. A positive anabolic effect of glutamine does not appear to be species-specific as it has already been demonstrated in rat skeletal muscle (MacLennan *et al.*, 1987). Since

glutamine also stimulates chick skeletal muscle protein synthesis (Wu and Thompson, 1990), the phenomenon is clearly a more general one and not confined to mammalian muscle tissue alone.

Previous studies showing a positive relationship between skeletal muscle glutamine concentration and protein synthesis *in vivo* have done so in disease or post-traumatic states (Jepson *et al.*, 1988; Hammarqvist *et al.*, 1989), with the intramuscular glutamine concentration ranging from low to physiological. To our knowledge this study shows for the first time that increasing plasma glutamine concentration can cause an increase in its intramuscular concentration in *healthy* subjects. Furthermore, the positive association between raised intramuscular glutamine concentration and muscle protein synthesis previously demonstrated in studies on animal muscle *in vitro* (MacLennan *et al.*, 1987; Wu and Thompson, 1990) and *in vivo* (Jepson *et al.*, 1988) is demonstrated *in vivo* in healthy man.

The mechanism by which glutamine stimulates skeletal muscle protein synthesis is not explained by this study. An indirect effect, possibly hormonally mediated, cannot be excluded as investigation of possible changes in plasma hormones accompanying Ala-Gln infusion was not undertaken. None of the amino acids alanine, glycine and glutamine are insulinogenic (Goodner and Porte, 1972) but no such information is currently available for the dipeptide Ala-glutamine. It has been suggested by Millward *et al* that hormones such as insulin may influence the relationship between intramuscular glutamine concentration and muscle protein synthesis in disease states (Jepson *et al.*, 1988; Millward *et al.*, 1989). Whether such influence is exerted in health, by hormones such as insulin, growth hormone and IGF-1, merits further study.

A further possibility of an indirect mechanism arises from the unexpected

finding of the intramuscular concentration of leucine and isoleucine being significantly greater in the Ala-Gln treated group. Leucine, in particular, has been reported to stimulate rat skeletal muscle protein synthesis *in vitro* (Buse and Reid, 1975). Considering the data available from this study, the possibility of a similar effect occurring in M. tibialis anterior in man, as an indirect consequence of Ala-Gln infusion and hence increased glutamine availability, cannot be excluded.

A direct stimulatory effect of glutamine on protein synthesis has been more readily demonstrated in *in vitro* studies (MacLennan *et al.*, 1987; Wu and Thompson, 1990). The subcellular mechanisms involved in this effect are poorly understood. Tyobeka has shown that despite the low K_m of glutamine for the aminoacyl *t*-RNA transferase reaction, glutamine is able to stimulate incorporation of labelled amino acids into protein in cell-free systems to a greater extent than other amino acids (Tyobeka and Manchester, 1985; Tyobeka, 1983). A possible explanation proposed by Tyobeka is that the availability of aminoacyl *t*-RNA synthetases for glutamine is dependant upon the presence of glutamine and this may be a regulatory effect.

Although there were no significant differences between the groups in whole-body protein turnover there was nevertheless a trend toward a lowering of whole-body protein breakdown with Ala-Gln provision. Further investigation with a larger number of subjects is merited to establish whether or not infusion of Ala-Gln has an acute effect on whole-body protein turnover in healthy man.

In conclusion, this study demonstrates that infusion of the dipeptide Ala-Gln in man raises glutamine concentrations in plasma and in muscle. The increased glutamine availability in muscle *per se* appears to stimulate muscle protein synthesis, calculated on the basis of the enrichments of plasma α-KIC. Since Ala-

Gln infusion does not appear to affect whole-body protein turnover to any large extent it is likely that in the absence of availability of dietary amino acids, skeletal muscle protein synthesis is increased by glutamine at the expense of non-muscle protein, as the muscle free amino acid pool could not sustain such an increase for more than a few minutes. Further studies of muscle protein breakdown (e.g. by using limb arteriovenous exchange techniques (Bennet *et al.*, 1990) may help to decide whether or not glutamine infusion has a net anabolic effect on skeletal muscle protein in healthy man.

CHAPTER 4

EFFECT OF INFUSION OF THE DIPEPTIDE L-ALANYL-L-GLUTAMINE ON SKELETAL MUSCLE PROTEIN SYNTHESIS AND BREAKDOWN IN HEALTHY POST-ABSORPTIVE MAN BY FOREARM [^{15}N]PHENYLALANINE ARTERIOVENOUS EXCHANGE

CONTENT

TABLES

FIGURES

4.1 SUMMARY

In this study the acute effects of L-alanyl-L-glutamine (Ala-Gln) dipeptide infusion on protein synthesis and breakdown in human forearm mixed skeletal muscle have been investigated. Fifteen healthy male post-absorptive subjects were studied: seven subjects received an infusion of Ala-Gln (11.67 mg/kg/h) and eight subjects received an isonitrogenous infusion of L-alanine (Ala, 4.78 mg/kg/h) plus glycine (Gly, 8.03 mg/kg/h) (Ala+Gly). Forearm skeletal muscle protein synthesis and breakdown were measured using a constant infusion of [^{15}N]phenylalanine with determination of arterialised venous plasma [^{15}N]phenylalanine kinetics and forearm arteriovenous exchange.

Arterialized venous blood glutamine concentration was higher during Ala-Gln than Ala+Gly infusion (640±40 (448, 948) *versus* 563±22 (506, 620) μmol/l, respectively, mean±SEM (95% confidence intervals), p=0.03).

Forearm muscle protein synthesis as determined by [^{15}N]phenylalanine exchange was 16% higher in the Ala-Gln group (30.4±2 (25, 36) *versus* 26.1±2 (21, 32) nmol/100g/min) although the difference was not statistically significant. Also, muscle protein breakdown was similar in each group (39.8±3 (33, 46) *versus* 35.6±3 (27, 44) nmol/100g/min). 3-methylhistidine balance was similar (-1.12±0.2 (-1.7, -0.5) *versus* -1.27±0.2 (-1.9, -0.7) nmol/100g/min) between the groups.

Whole body phenylalanine flux values were similar in the two groups (34.7±1.1 (32, 38), Ala-Gln *versus* 39.2±2.0 (34, 44) Ala+Gly, μmol/kg/h), although as with the previously described whole-body leucine flux (Chapter 3), there was a trend toward lower values in the Ala-Gln group.

These results suggest that in healthy man increased availability of

glutamine, provided as the dipeptide Ala-Gln, does not acutely stimulate muscle mixed protein synthesis, as assessed by [^{15}N]phenylalanine arteriovenous exchange nor does it alter the rate of protein breakdown in the post-absorptive state.

4.2 Introduction

Injury, including surgical trauma, can result in net whole-body negative nitrogen balance. Following major surgery skeletal muscle wasting occurs, resulting in efflux of a number of amino acids (Rennie, 1985) including glutamine, the most abundant free amino acid in human skeletal muscle (Fürst, 1983). This results in a 50-60% reduction in the intramuscular free glutamine pool (Vinnars *et al.*, 1975; Askanazi *et al.*, 1980a, 1980b; Roth *et al.*, 1982; Fürst, 1984) and is associated with a fall in skeletal muscle protein synthesis as measured indirectly using intramuscular polyribosome concentration (Hammarqvist *et al.*, 1989). In animal studies a positive correlation has been demonstrated between intramuscular glutamine concentration and muscle protein synthesis (Jepson *et al.*, 1988). Provision of glutamine to post-surgical patients results in a diminution in the fall in intramuscular glutamine concentration and also reduces the extent of the negative nitrogen imbalance (Hammarqvist *et al.*, 1989; Stehle *et al.*, 1989). Hammarqvist *et al.* (1989) have demonstrated that administration of a total parenteral nutrition (TPN) solution supplemented by glutamine, to patients undergoing elective abdominal surgery reduces the fall in intramuscular free glutamine, improves nitrogen balance and counteracts the fall in muscle polyribosome concentration. Information from both *in vivo* and *in vitro* animal

studies would appear to suggest that glutamine inhibits protein breakdown in cultured rat skeletal muscles (Smith, 1985) and in the perfused rat hindlimb (MacLennan *et al.*, 1989). In an *in vitro* study Wu and Thompson demonstrated that glutamine decreases protein breakdown in chick skeletal muscle (Wu and Thompson, 1990). Although these studies have provided valuable information in forming a more detailed picture of glutamine's effect on skeletal muscle protein balance no similar studies have been reported in man. Because of major differences in skeletal muscle composition and in particular the free glutamine pool size in man (Fürst *et al.*, 1987) information obtained from animal studies on glutamine's role in muscle protein metabolism may have only limited application to man. Provision of parenterally administered glutamine in a stable form as the dipeptide L-alanyl-L-glutamine (Ala-Gln) (Stehle *et al.*, 1984; Fürst *et al.*, 1987; Albers *et al.*, 1988; Albers *et al.*, 1989) increases glutamine availability, reduces post-operative muscle loss of alanine and glutamine (Roth *et al.*, 1988) and whole-body N-excretion (Stehle *et al.*, 1989).

From our previous study (see Chapter 3) although Ala-Gln appears to have a small but significant stimulatory effect on skeletal muscle protein synthesis in healthy post-absorptive man as determined by tracer [1-^{13}C]leucine incorporation studies, such an investigative approach is unable to provide information on muscle protein breakdown. We have therefore investigated the possible acute effects of Ala-Gln infusion on muscle protein synthesis *and* breakdown in 15 healthy male post-absorptive subjects using a forearm arteriovenous phenylalanine exchange method with [^{15}N]phenylalanine as tracer.

This technique may be used to measure arteriovenous uptake and release of phenylalanine across a limb (Gelfand and Barrett, 1987; Thompson *et al.*, 1989;

Cheng *et al.*, 1985), which predominantly contains skeletal muscle (Häggmark *et al.*, 1978). In skeletal muscle the only metabolic fates of phenylalanine are incorporation into protein and release from protein; catabolism of phenylalanine to tyrosine occurs predominantly in liver and does not occur in muscle. Uptake and release of phenylalanine by the limb are therefore, indices of protein synthesis and breakdown respectively. The ^{15}N enrichment of arterial and venous plasma phenylalanine, during a primed continuous infusion of [^{15}N]phenylalanine, and plasma concentrations may be used to calculate phenylalanine exchange by limb tissue from the degree of dilution of the tracer in the venous outflow in comparison with the arterial inflow.

4.3 METHODS

4.3.1 Subjects

The present studies were performed concurrently with the studies described in Chapter 3 and the same subjects were studied. As described in Chapter 3, the fifteen healthy male subjects (age 24-52 y, weight 63.8-84.5 kg, Table 3.1, Chapter 3) were studied in the Department of Surgery, Ninewells Hospital and Medical School, Dundee University. Each subject gave written consent after a full explanation of the study. The protocol for the study was approved according to the Declaration of Helsinki guidelines by the Dundee General Hospitals Committee on Medical Ethics of the Tayside Health Board.

4.3.2 Study Design

Subjects were randomised to receive either Ala-Gln infusion (n=7) or isonitrogenous L-alanine *plus* glycine (Ala+Gly, n=8). After an overnight 15 h fast, subjects were studied in the supine position in a thermoneutral environment (25-27^{o}C). No food or water was given to the subjects until the study was complete. Whole-body tracer phenylalanine turnover and forearm muscle protein balance as determined by [^{15}N]phenylalanine arteriovenous exchange were investigated over an 8 h period in each group.

4.3.3 Materials

Solutions of L-[$^{[15}$N]phenylalanine (99 atoms %, Tracer Technologies Inc., Newton, MA, U.S.A.) were prepared under aseptic conditions in sterile, pyrogen-free 0.9% (150 mmol/l) sodium chloride solution (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.). The solutions were sterilized by filtration through 0.22 μm filters (Millipore SA, Molsheim, France). Solutions were similarly prepared of the dipeptide Ala-Gln (Kabi-Pfrimmer, Erlangen, Germany) and a mixture of the unlabelled amino acids L-alanine and glycine (Sigma Laboratories, U.K.).

4.3.4 Study Details

Arterialized venous blood ('arterial' blood) sampling was carried out via a 1.7

mm outer diameter cannula (Venflon, Viggo AB, Helsingborg, Sweden), placed retrogradely in a dorsal hand vein and maintained patent by slow infusion of 150 mmol/l NaCl solution. Before each sampling the hand was warmed for 10 min by insertion in a thermostatic chamber heated to 65°C (Abumrad, *et al.*, 1981). A second cannula was placed antegradely in an antecubital vein of the ipsilateral arm for tracer amino acid and dipeptide infusions. The third cannula was inserted retrogradely in an antecubital vein of the contralateral forearm for venous blood sampling. Priming doses of L-[^{15}N]phenylalanine (0.5 mg/kg) were administered (Matthews *et al.*, 1980; Nair, *et al.*, 1988) followed by a constant infusion of tracer L-[^{15}N]phenylalanine (0.5 mg/kg/h) with Ala and Gly (4.78 and 8.03 mg/kg/h respectively, control group) or L-[^{15}N]phenylalanine with Ala-Gln (11.67 mg/kg/h, study group) maintained and delivered over 8 h with an IMED 928 volumetric infusion pump (IMED Ltd, Abington, U.K.). Forearm blood flows were measured by venous occlusion mercury-in-silastic strain-gauge plethysmography (Medimatic, Denmark) at regular intervals throughout the study period, on each occasion 2 minutes prior to blood sampling. During flowrate measurements as well as during blood sampling, a paediatric sphygmomanometer cuff was inflated to above systolic pressure at wrist level of the study forearm to temporarily occlude and exclude the hand circulation from measurements (Jackson *et al.*, 1987). Paired arterialised venous and venous blood samples were taken before the start of the tracer infusion and at intervals throughout the study (30, 90, 150, 240, 330, 420, 480 min) for blood gas analysis (P_{CO_2}, P_{O_2}, SaO_2% - Corning 178 Blood Gas Analyzer, CIBA Corning Diagnostics, Essex, U.K.), amino acid concentrations and for determination of [^{15}N] enrichment of (plasma) phenylalanine.

4.3.5 Analysis of Amino Acid Concentrations and Phenylalanine [^{15}N] Enrichment

The methods of analysis used in the present study have been detailed in Chapter 2. Plasma [^{15}N] enrichment of phenylalanine was determined by gas chromatography-mass spectrometry (Hewlett-Packard Ltd., Cheadle Heath, U.K.) (Rocchiccioli *et al.*, 1981). Free amino acid concentrations were determined in plasma following sulphosalicylic acid extraction with an amino acid analyzer (LC5000, Biotronik GmbH, Munchen, Germany) using ion-exchange chromatography and fluorometric detection of *o*-phthaldehyde derivatives with norvaline as internal standard.

4.3.6 Calculations

The ^{15}N enrichment of arterial and venous plasma phenylalanine during the primed continuous infusion of [^{15}N]phenylalanine and plasma concentrations of phenylalanine were used to calculate phenylalanine exchange by limb tissue from the degree of dilution of the tracer in the venous outflow in comparison with the arterial inflow. The equations used are detailed in Chapter 2. The components of plasma phenylalanine turnover were calculated from the mean arterialised venous and venous phenylalanine enrichment and concentration from 330 to 480 min.

4.3.7 Statistical Analysis

Intergroup differences in results were analyzed with the Student's *t*-test for non-paired data (two-tailed, except where otherwise stated) whilst comparison of intra-group differences were made using Student's *t*-test for paired data (two-tailed, except where otherwise stated). 95% confidence intervals are also given in the table and in the text.

4.4 RESULTS

4.4.1 Plasma Concentration and [^{15}N] Enrichment of Phenylalanine

Plateau was attained for the enrichment of arterial plasma phenylalanine during the period 90-480 min of each study (Figure 4.1). There were no significant differences in enrichment of phenylalanine in the period 90-480 min between the two groups in either arterial (APE: 7.10$\pm$0.3 (6.4, 7.8), Ala+Gly *versus* 7.86$\pm$0.3 (7.2, 8.5), Ala-Gln) or venous (APE: 5.87$\pm$0.3 (5.1, 6.6), Ala+Gly *versus* 6.13$\pm$0.3 (5.3, 6.9), Ala-Gln) plasma.

4.4.2 Plasma Free Amino Acid Concentrations

The concentrations of free amino acids in arterial plasma, and the changes in their concentrations from their basal values to values attained at plateau in

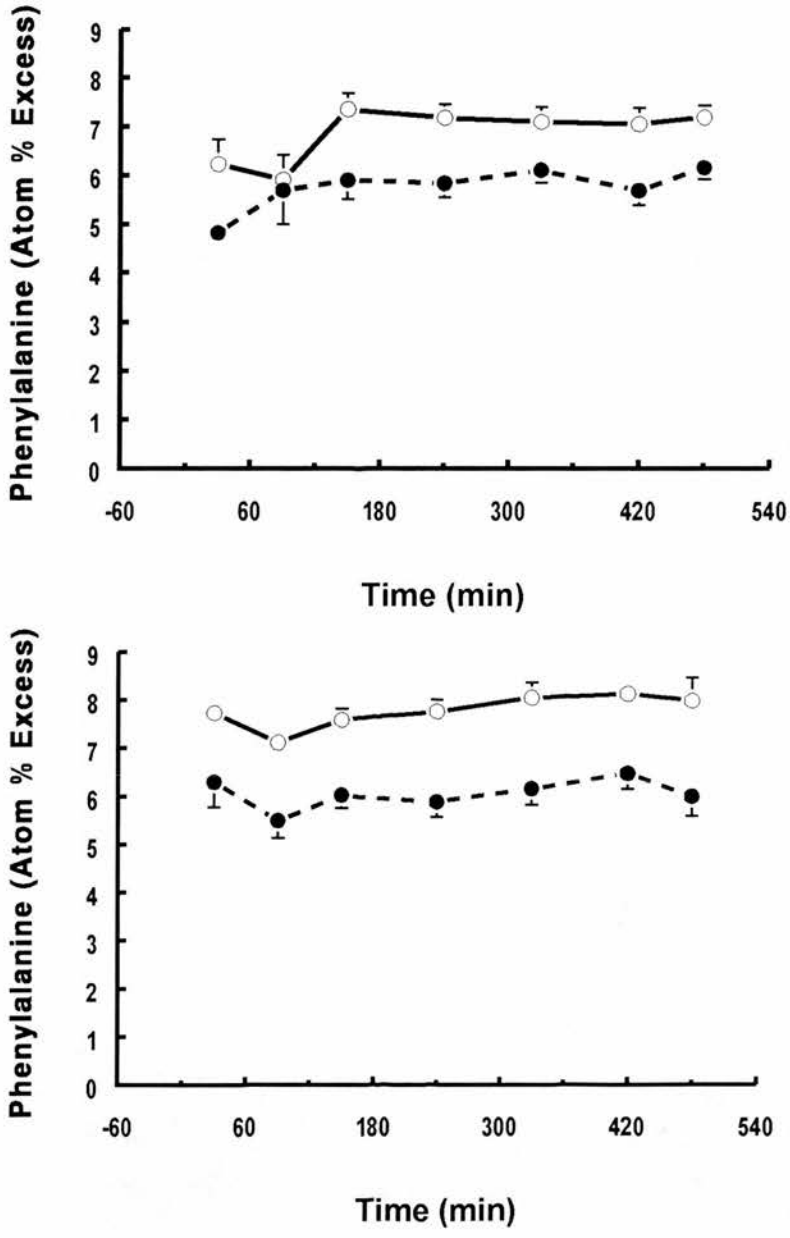

Figure 4.1 Plasma arterial (O) and venous (●) enrichment of free phenylalanine in the Ala+Gly group (top graph) and Ala-Gln group (bottom graph).

either the Ala+Gly group or the dipeptide infused group are discussed in part in chapter 3. The basal arterial plasma glutamine concentration in the Ala+Gly group was 555±22 (498, 611) μmol/l (mean±SEM (95% CI)) and 563±22 (506, 620) μmol/l during the period 90 to 480 min, thus showing little change during the study period (Table 3.2 and Figure 3.1 , Chapter 3). In the Ala-Gln group the basal glutamine concentration was 566±36 (476, 651) μmol/l, rising (p=0.03) to a plateau concentration of 640±43 (534, 746) μmol/l, between 90 to 480 min. The concentrations of the amino acids L-leucine, L-tyrosine and L-glutamic acid also changed from their basal values in both groups, although there was no significant difference in the values between the two groups. The plasma concentration of phenylalanine was stable during the period 90 to 480 min in each group (Figure 4.2). The basal arterial plasma phenylalanine concentration in the Ala+Gly group was 50.6±4 (41, 60) μmol/l rising slightly to 52.8±4 (44, 61) μmol/l (p=0.07) during the period 90 to 480 min. In the Ala-Gln group the basal phenylalanine concentration was 49.5±2 (44, 55) μmol/l, showing a small rise of 7% to 52.9±2 (46, 59) μmol/l between 90 to 480 min. There were small statistically insignificant falls in the concentrations of 3-methylhistidine in each group from basal to plateau values (4.84±0.6 (3.3, 6.4) to 4.15±0.5 (3.0, 5.3) μmol/l Ala+Gly group; 5.25±0.6 (3.8, 6.7) to 4.14±0.4 (3.1, 5.2) μmol/l in the Ala-Gln group), the 90-480 min values being identical between the two groups.

4.4.3 Forearm Amino Acid Balance

The net effluxes from the forearm of phenylalanine and tyrosine in the

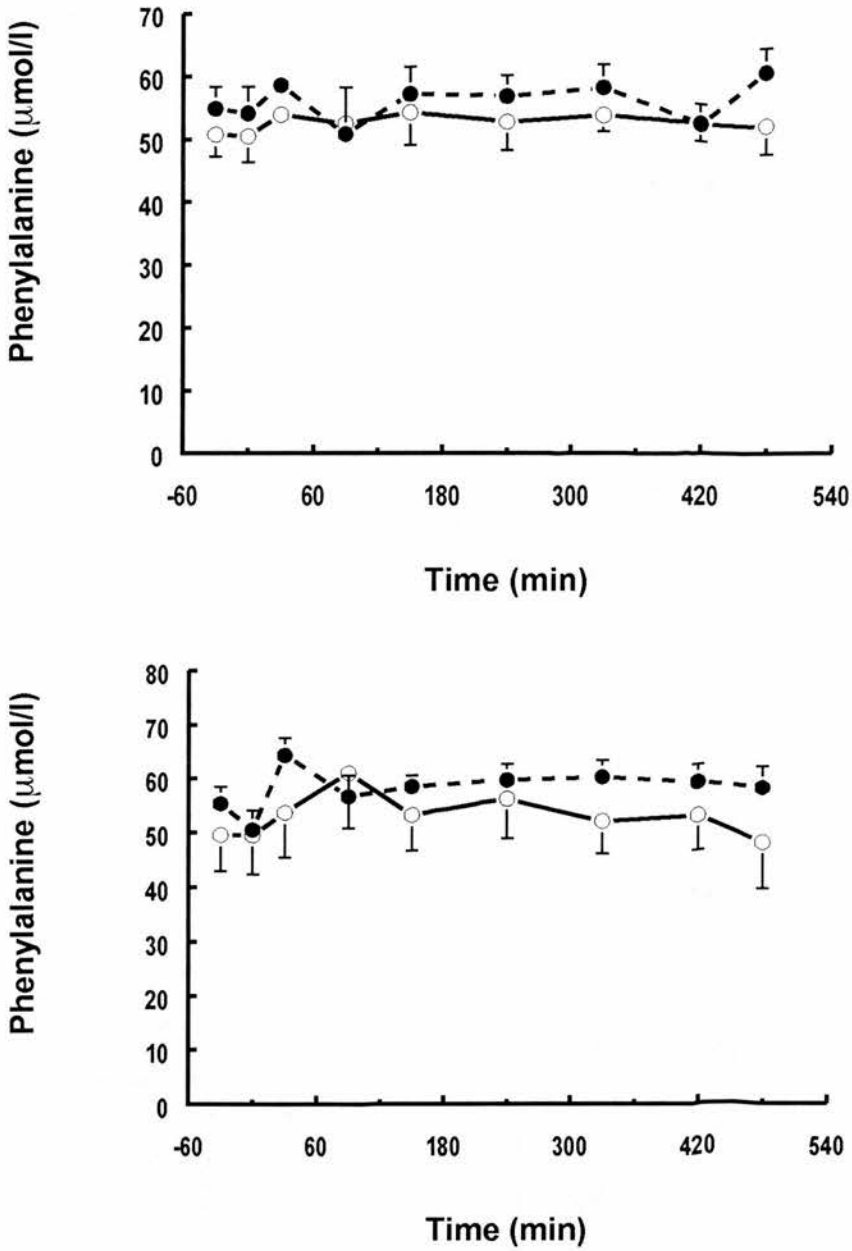

Figure 4.2 Plasma arterial (O) and venous (●) concentrations of free phenylalanine in the Ala+Gly group (top graph) and Ala-Gln group (bottom graph).

Ala+Gly group at plateau were -11.5±3 (-24, -5) and -12±3 (-20, -5) nmol/100g/min respectively (Table 4.1). The corresponding efflux values for phenylalanine and tyrosine in the Ala-Gln group were similar at -11.4±2 (-15, -8) and -16±3 (-25, -7) nmol/100g/min. The efflux of 3-methylhistidine from the forearm during 90-480 min was 13% higher in the Ala-Gln group (-1.27±0.2 (-1.9, -0.7) *versus* -1.12±0.2 (-1.7, -0.5) nmol/100g/min respectively), although this difference was not statistically significant, suggesting that there was no significant difference in the rates of myofibrillar protein breakdown between the two groups.

4.4.4 Forearm Protein Turnover by [^{15}N]Phenylalanine Arteriovenous Exchange

Forearm mixed protein breakdown, assessed by [^{15}N]phenylalanine exchange, was 35.6±4 (27, 44) nmol/100g/min in the Ala+Gly group. Although the rate was higher in the Ala-Gln group at 39.8±3 (33, 46) nmol/100g/min the difference was not significant (p=0.603) (Figure 4.3). Forearm protein synthesis was 26.1±2 (21, 32) nmol/100g/min in the control group and was 16% higher at 30.4±2 (25, 36) nmol/100g/min (p=0.09, 1-tailed) in the Ala-Gln group. The balance between protein synthesis and protein breakdown was very similar between the two groups at -11.5±3 (-24, -5) (Ala+Gly) and -11.4±2 (-15, -8) (Ala+Gln) nmol/100g/min.

Table 4.1 Plasma arteriovenous amino acid balance. BCAA, total branched chain amino acids, i.e. the sum of leucine, isoleucine and valine. Results are means±SEM. CI = 95% confidence intervals.

	Amino acid balance (nmol/100g/min)							
	Ala+Gly				Ala-Gln			
	Basal	C.I.	90-480 min	C.I.	Basal	C.I.	90-480 min	C.I.
ALA	-144±33	(-223, -66)	-117±29	(-189, -45)	-152±13	(-186, -117)	-112±32	(-194, -30)
GLU	121±13	(88, 153)	90±14	(52, 128)	114±14	(79, 148)	94±13	(53, 134)
GLN	-274±53	(-424, -125)	-295±42	(-397, -194)	-261±27	(-330, -193)	-304±16	(-346, -262)
GLY	-110±50	(-228, 7)	13±24	(-45, 71)	-108±30	(-181, -35)	-96±18	(-140, -52)
3-MH	-0.90±0.5	(-2.1, 0.3)	-1.12±0.2	(-1.7, -0.5)	-1.00±0.1	(-1.3, -0.7)	-1.27±0.2	(-1.9, -0.7)
ISO	-13±6	(-27, 0.5)	-10±5	(-20, -1.2)	-15±1	(-17, -13)	-16±4	(-25, -7)
LEU	-27±11	(-51, -2)	-21±10	(-45, 3)	-28±2	(-33, 22)	-31±6.5	(-48, -14)
PHE	-14±4	(-25, -3.3)	-12±3	(-24, -5)	-15±2	(-21, -9)	-11±2	(-15, -8)
TYR	-16±4	(-27, -5)	-12±3	(-20, -5)	-16±1	(-19, -12)	-16±3	(-25, -7)
VAL	-23±9	(-46, -0.4)	-22±10	(-46, 3)	-21±6	(-38, -4)	-26±8	(-47, -4)
BCAA	-41±16	(-80, -3)	-53±21	(-104, 3)	-48±12	(-78, -18)	-59±17	(-100, -16)

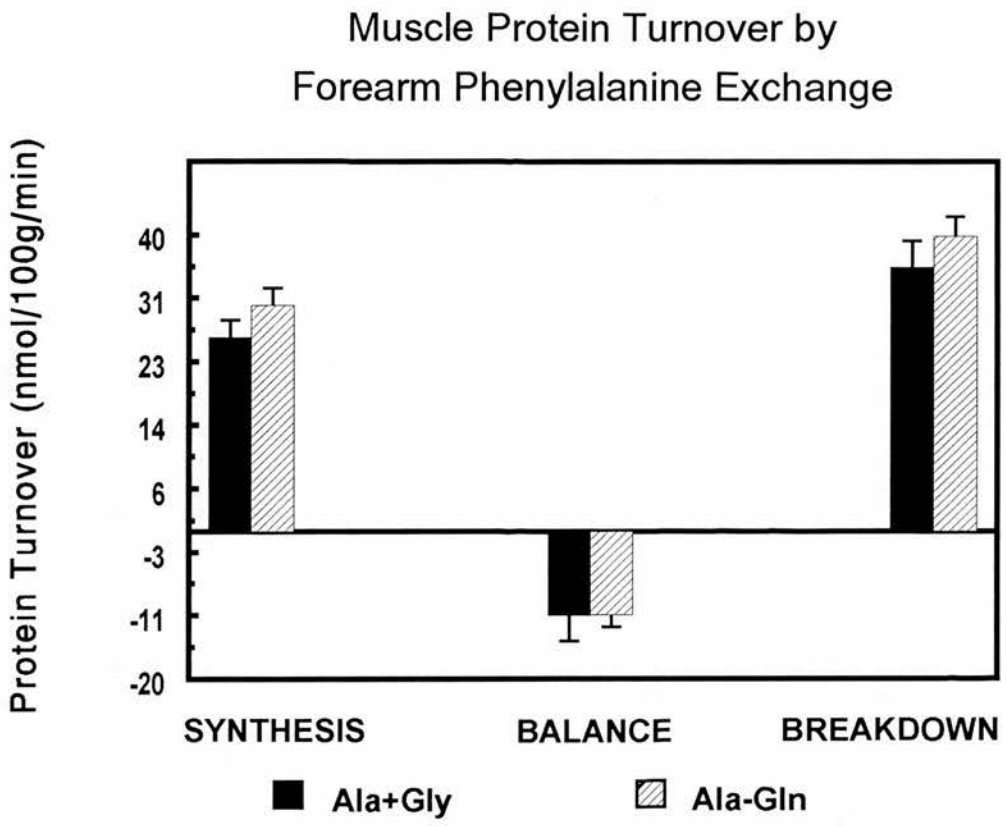

Figure 4.3 Components of forearm phenylalanine metabolism in the Ala+Gly group (solid bars) and Ala-Gln group (hatched bars).

4.4.5 Calculated Forearm Skeletal Muscle Fractional Protein Synthetic Rate

With the information that muscle contains 18% protein, protein contains 4% phenylalanine (Gelfand and Barrett, 1987), and assuming on the fat content of the subjects, and that the forearm is representative of the whole-body (Maughan *et al.*, 1984), that 75% of forearm blood flow is distributed to muscle at rest, the fractional protein synthetic rate may be calculated as 0.048±0.004 (0.038, 0.058) %/h in the control group (Figure 4.4) and 0.052±0.005 (0.039, 0.065) %/h ($p<0.09$, 1-tailed) in the Ala-Gln group and this difference between the two groups was not significant.

4.4.6 Whole-body Phenylalanine Kinetics

There were no significant differences between the two groups in whole-body phenylalanine kinetics (Figure 4.5). Whole-body phenylalanine flux was 39.2±2 (34, 44) μmol/kg/h in the Ala+Gly group and was 11% lower in the Ala-Gln group at 34.7±1 (32, 38) μmol/kg/h ($p=0.105$). Whole-body protein breakdown was 36.2±2 (31, 41) μmol/kg/h in the control group and was 12% lower in the Ala-Gln group at 31.7±1 (29, 34) μmol/kg/h but this difference was not significant ($p=0.105$).

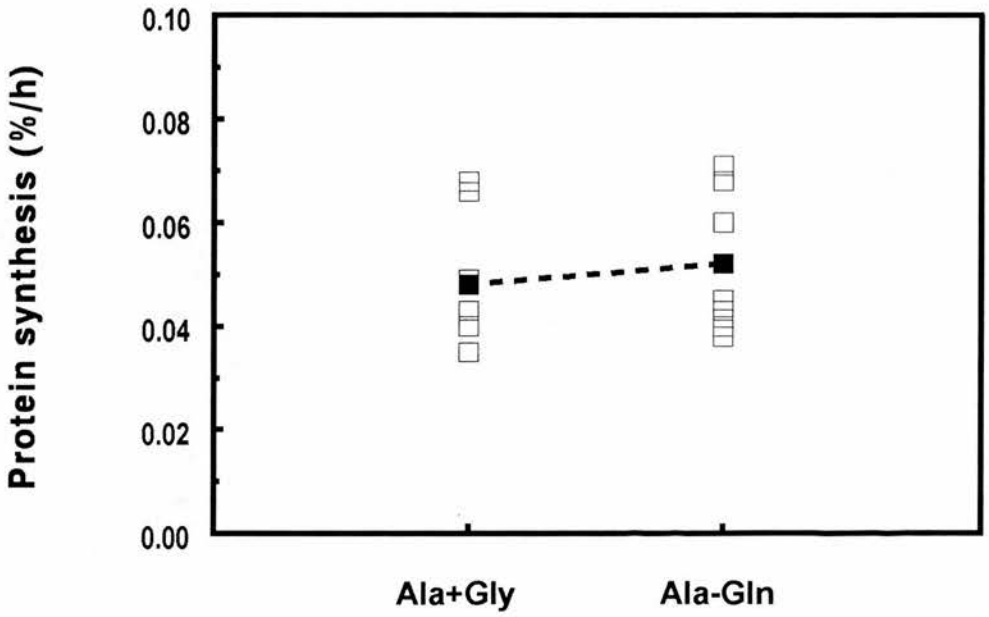

Figure 4.4 Skeletal muscle fractional mixed protein synthesis in the Ala+Gly and Ala-Gln groups (hollow squares: individual values; solid squares: mean values).

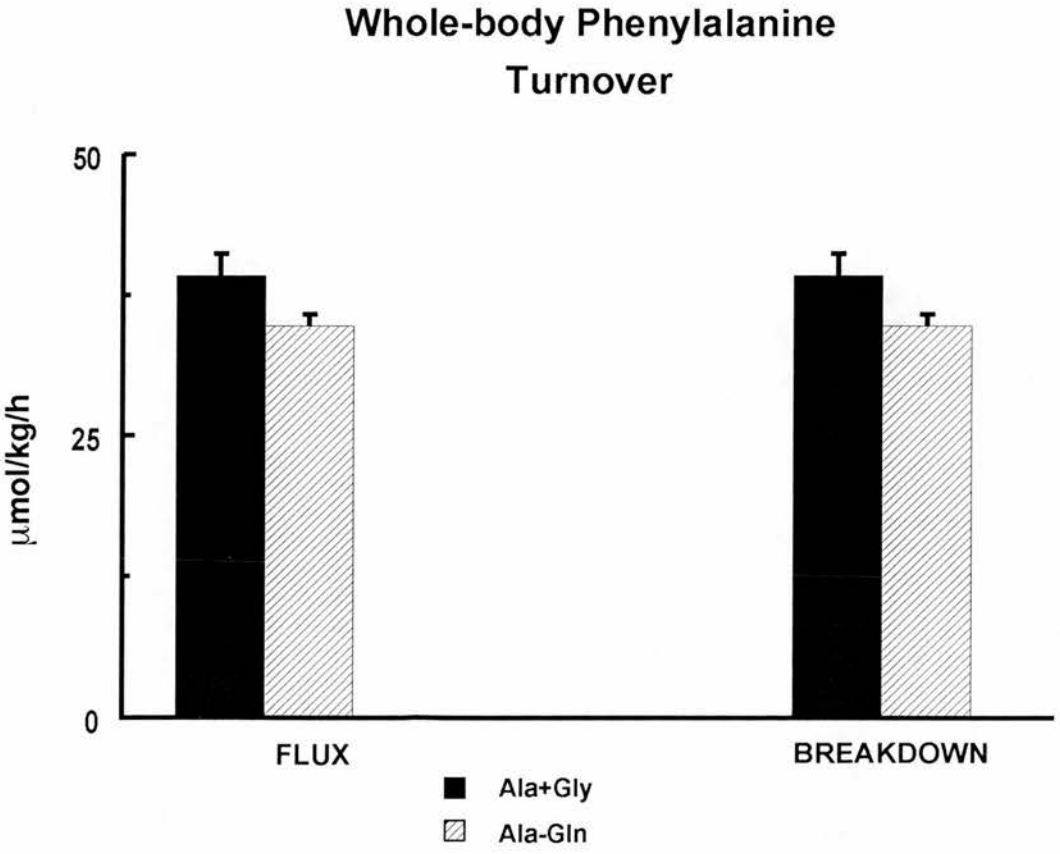

Figure 4.5 Whole-body phenylalanine kinetics (flux and breakdown) in the Ala+Gly group (solid bars) and Ala-Gln group (hatched bars).

4.5 DISCUSSION

In this study, parenteral administration of the dipeptide Ala-Gln, raised arterialized venous blood glutamine concentration in the study group by 18 % ($p<0.05$) and this latter value was also significantly higher ($p=0.03$) than during Ala+Gly infusion. Thus, for the first time, we were able to evaluate the effects of glutamine provision on muscle protein synthesis *and* breakdown in healthy post-absorptive man. There is good evidence from previously reported studies (Albers *et al.*, 1986; Albers *et al.*, 1988), that in healthy subjects there is both rapid and complete hydrolysis of the dipeptide Ala-Gln once infused, releasing the free amino acids alanine and glutamine. Also, a similar finding has been reported in post-operative patients (Stehle *et al.*, 1989) in whom there was no detectable Ala-Gln dipeptide (as determined by high performance liquid chromatography) in the arterial plasma during constant infusion of the dipeptide in amounts equivalent to the dose used in the present study. In the present study, therefore, as both groups received equivalent amounts of alanine, the comparison between the groups, in essence, was between glutamine and glycine, with glycine being used as the control amino acid. The alanine and glycine mixture was given in isonitrogenous amounts to the Ala-Gln dipeptide to eliminate the effect of the variable total amino-nitrogen on muscle protein synthesis.

In designing the study, without prior information available as to the likely possible effect, either qualitatively or quantitatively, of glutamine on muscle protein synthesis and breakdown in man, two separate subject groups were used. The subject numbers are relatively small and the practical

difficulties of recruiting and performing fairly invasive and long metabolic studies as described have placed some constraint on the subject numbers. Ideally, if such constraints were not in place, it may have been possible and desirable to repeat the studies in each subject in a 'cross-over' manner so that the control subjects, receiving Ala+Gly, would receive Ala-Gln in the second study and *vice versa*. Such a study design, with the subjects being self-controlled, would have made statistical comparison more powerful by minimising the considerably greater effects of biological variation in response to nutrient provision observed in man in comparison to laboratory animals.

The infusion rate of Ala-Gln chosen for this study (11.67 mg/kg/h) was the highest that had previously been shown to be safe and effective in raising plasma Gln concentrations in man (Stehle *et al.*, 1989). However, in that study, performed in post-operative subjects, this dose was calculated to *replace* the expected post-operative loss of nitrogen. This dose was therefore in the pharmacological range and by using a similar dose in healthy man in the present studies we were in effect investigating the effects of supra-physiological glutamine provision in the post-absorptive state.

An additional variable in this present study was the effect of blood flow measurements on the calculated values of amino acid exchange. Small changes in blood flow can significantly affect the measured net balance of a metabolite across a limb. Consequently, errors in blood flow measurement may contribute the largest proportion to the overall error of arteriovenous exchange methodology (Butler and Home, 1987; also, see equation, Chapter 2, section 2.5.2) and every effort was made to minimise error and maintain consistency in technique by using an identical method to measure blood flow in all subjects.

The laboratory analytical techniques used for measurement of plasma amino acid concentrations and [^{15}N]phenylalanine enrichment were already well-established in our centre and reported elsewhere (Bennet *et al*, 1989) and were felt to be appropriate for this study. Previously established analysis protocols were strictly adhered to in order to minimise laboratory error.

The rates of forearm muscle protein synthesis and breakdown in the Ala+Gly (control) group are of a very similar order to previously published values from our centre, for skeletal muscle synthesis (29$\pm$5 nmol/100g/min) and breakdown (42.8$\pm$4 nmol/100g/min), in post-absorptive healthy subjects (Bennet *et al.*, 1989) using identical tracer methodology (i.e. using the arteriovenous limb exchange of [^{15}N]phenylalanine), although the leg preparation was used for those studies rather than the forearm as described here. Thompson and colleagues (Thompson *et al.*, 1989), using deuterated phenylalanine and the forearm preparation, have also reported similar values for synthesis (37 nmol/100g/min) and breakdown (46 nmol/100g/min). Gelfand and Barrett (1987) have used the tracers tritiated phenylalanine and [^{14}C]leucine and the forearm preparation to determine values of 43 and 57 nmol/100g/min for synthesis and breakdown respectively with the former tracer and much higher values of 113 and 126 nmol/100g/min respectively with the latter tracer. Cheng and colleagues (Cheng *et al.*, 1987) have also used the forearm preparation but have used the stable isotope tracer [^{13}C, ^{15}N]leucine to obtain values of 70 and 98 nmol/100g/min respectively for muscle protein synthesis and breakdown.

In the post-absorptive state in healthy man, glutamine provision, in the form of the dipeptide Ala-Gln, does not appear to reduce the rate of muscle protein breakdown. This result is at variance with that of others who have

reported an inhibitory effect of glutamine on muscle protein breakdown (MacLellan *et al.*, 1989; Wu and Thompson, 1990). This may be explained in large part by i) the different methodologies employed, with use by McLelland *et al.*, (1989) of an isolated perfused rat hindlimb and the use of isolated chick muscle by Wu and Thompson (1990). Also, interspecies differences in muscle protein metabolism particularly and their bearing on muscle glutamine metabolism are incompletely understood; they may also account for the different response to glutamine provision in muscle protein breakdown observed in man in the present study.

In this study, we were unable to demonstrate a statistically significant effect on protein synthesis of Ala-Gln administration, as determined by arteriovenous [^{15}N]phenylalanine forearm exchange, in contrast to the earlier study (described in Chapter 3) where protein synthesis was higher in the Ala-Gln group. In part this is likely to be explained by the different muscles under study i.e., M. tibialis anterior in the incorporation study and the muscles of the forearm in the [^{15}N]phenylalanine exchange study. It is known that skeletal muscles differ in their rates of protein synthesis (Smith and Rennie, 1990) and the variation in the acute response of skeletal muscle protein synthesis to Ala-Gln provision seen in these studies may well in part be accounted by this factor alone. Additionally, the forearm exchange method for determining rates of protein synthesis is possibly less sensitive than the tracer incorporation method and any actual small difference between the two groups would arguably be more likely to be detected by the latter method than the former (Biolo *et al.*, 1995).

Protein synthesis was higher in the dipeptide-infused group and although the difference was not statistically significant, qualitatively the difference is

similar in direction to that observed in the previous study on the effect of Ala-Gln on muscle protein synthesis as determined by [1-^{13}C]leucine incorporation. The findings of this study, taken in consideration with those of the earlier study, would appear to suggest a possible mild acute stimulatory effect of glutamine provided as the dipeptide on skeletal muscle protein synthesis but with no effect on muscle protein breakdown, in healthy post-absorptive man.

CHAPTER 5

THE EFFECT OF L-ALANYL-L-GLUTAMINE SUPPLEMENTATION OF CONVENTIONAL TPN ON SKELETAL MUSCLE PROTEIN SYNTHESIS IN POST-SURGICAL PATIENTS: STUDIES OF [1-^{13}C]LEUCINE INCORPORATION INTO MUSCLE PROTEIN

CONTENTS

TABLES

FIGURES

5.1 SUMMARY

The aim of this study was to investigate the acute effects of L-alanyl-L-glutamine (Ala-Gln) dipeptide supplementation of conventional TPN on skeletal muscle protein synthesis in post-surgical patients receiving conventional glutamine-free total parenteral nutritional support after major surgery. Sixteen patients received a constant infusion of non-glutamine containing TPN from the first post-operative day up to and including the third post-operative day when muscle protein synthesis was investigated over an eight hour period using a primed constant infusion of [1-^{13}C]leucine with determination of plasma leucine-C flux and assay of [1-^{13}C]leucine incorporation into mixed anterior tibial muscle protein sampled by biopsy. The effects of TPN alone were studied in eight of sixteen patients (cross-sectional study of Ala-Gln supplementation); the remaining eight patients were studied over two 4-hour periods (longitudinal study of Ala-Gln supplementation). In the first, TPN was supplemented with a primed constant infusion of L-alanine plus glycine (Ala+Gly) as a control mixture isonitrogenous with the primed constant infusion of Ala-Gln given in the second period.

The mean basal (-30 to 0 min) plasma arterial glutamine concentration in the cross-sectional study was 332±17 (287, 377) μmol/l (mean±SEM, (95% confidence intervals)) and remained unchanged during 90-480 min at 340±11 (311, 370) μmol/l. The mean basal plasma arterial glutamine concentration in the longitudinal study was 330±16 (281, 364) μmol/l and 314±16 (274, 355) μmol/l during infusion with Ala+Gly, rising by 29% to 404±30 (316, 471) μmol/l ($p<0.05$) with the infusion of Ala-Gln. The intramuscular concentration of glutamine fell by 51% from the pre-operative value of 62±4 (51, 72) μmol/g protein to a post-operative

value of 30±3 (24, 37) μmol/g protein at the end of the Ala+Gly supplementation period ($p<0.0005$), rising by a small but significant amount to 34±3 (26, 42) μmol/g at the end of Ala-Gln infusion ($p<0.03$, 1-tailed).

For both plasma leucine and α-ketoisocaproate, plateau enrichment was attained during the last 180 min of each phase of the longitudinal study and during the last 390 min of the cross-sectional study. In the longitudinal study, the intracellular leucine enrichment remained unchanged (3.21±0.17 (2.8, 3.6) APE, Ala+Gly *versus* 3.24±0.11 (3.0, 3.5) APE, Ala-Gln). The fractional rates of protein synthesis in M. tibialis anterior with TPN alone, during TPN + Ala+Gly and during TPN + Ala-Gln, calculated on the basis of each of the two different surrogate precursor pools plasma α-ketoisocaproate and intracellular leucine were respectively: plasma α-ketoisocaproate: 0.031±0.003 (0.026, 0.036) %/h, 0.025±0.003 (0.016, 0.034) %/h, 0.047±0.006 (0.031, 0.062) %/h ($p=0.0052$, TPN + Ala-Gln *versus* TPN + Ala+Gly; $p=0.049$, TPN + Ala-Gln *versus* TPN alone); intracellular free leucine: 0.049±0.005 (0.037, 0.061) %/h, 0.039±0.003 (0.032, 0.047), 0.075±0.008 (0.055, 0.096) ($p=0.0095$, TPN + Ala-Gln *versus* TPN + Ala+Gly; $p=0.04$, TPN + Ala-Gln *versus* TPN alone). Whole-body leucine kinetics were similar during TPN alone and during TPN + Ala+Gly and TPN + Ala-Gln, although there was a trend towards lower values for leucine flux with TPN + Ala-Gln. These results suggest that short-term Ala-Gln supplementation of TPN in post-operative patients results in a modest repletion of the intramuscular free glutamine pool and acutely stimulates skeletal muscle mixed protein synthesis, assessed by [1-^{13}C]leucine incorporation into muscle protein, irrespective of the surrogate precursor pool (for *t*RNA) used to calculate protein synthesis.

5.2 Introduction

Following surgery particularly major surgery, or following trauma and sepsis intramuscular free glutamine falls by up to 60% (Vinnars *et al.*, 1975; Askanazi *et al.*, 1980a; Askanazi *et al.*, 1980b). Negative nitrogen balance occurs, associated with muscle wasting. Muscle wasting is likely to be significant in adding to post-operative mortality and morbidity, particularly in increasing complications such as chest infections, deep vein thrombosis and slow mobilisation. About 50% of patients undergoing major surgery are malnourished (Bistrian *et al.*, 1975). Complications associated with peri-operative malnutrition are compounded by a pre-operative malnutritional state. Malnutrition may result in delayed wound healing, increased frequency of wound infections, decreased vital organ function, decreased immunocompetence, increased occurrence of sepsis, prolonged ileus and more frequent pulmonary infections and pulmonary insufficiency (Mullen *et al.*, 1979; Reinhardt *et al.*, 1980; Seltzer *et al.*, 1979). Severe malnutrition is likely to occur in patients who have been unable, for a number of reasons, to maintain an adequate diet over a prolonged period of time pre-operatively. The malnourished state may be compounded in the post-operative period, particularly in patients with upper gastrointestinal pathology requiring major oesophageal and gastric surgery and who may be unable to take adequate nutrition orally, either through a clinical requirement to protect gastrointestinal anastomoses or because of post-operative small intestinal ileus. Conventional parenteral nutrition was established by the pioneering work of Dudrick *et al.* (1967, 1968). Although parenteral nutrition has not been shown to be of value in normally nourished or mildly nourished patients (Detsky *et al.*, 1987) it has shown been shown that perioperative TPN confers

significant benefits in terms of reducing post operative morbidity and mortality (Mullen *et al.*, 1980). However, conventional TPN, comprising a mixture of amino acids, carbohydrates (glucose) and lipids, does not appear to reverse post-operative negative nitrogen balance nor does it prevent post operative muscle wasting.

The observed post-operative fall in intramuscular glutamine is associated with a fall in skeletal muscle protein synthesis (Hammarqvist *et al.*, 1989). A positive correlation has been demonstrated between intramuscular free glutamine and muscle protein synthesis by Jepson *et al.* (1988). Provision of glutamine by the parenteral route appears to reduce the extent of the post-operative negative nitrogen balance (Stehle *et al.*, 1989). Although conventional TPN did not significantly alter post-operative muscle protein synthesis when compared to pre-operative values, studied using the flooding dose technique (Essén, 1991), glutamine supplementation appeared to raise post-operative muscle glutamine concentration and reduce the extent of the post-operative fall in muscle protein synthesis as assessed *indirectly* from the intramuscular polyribosome concentration as an index of protein synthesis (Hammarqvist *et al.*, 1989) in muscle. Because of reported potential problems with the solubility and possibly the stability of free glutamine solutions for heat sterilisation and storage (Stehle and Fürst, 1987) we have used the dipeptide L-alanyl-L-glutamine (Ala-Gln). This peptide has greater solubility and stability and upon intravenous infusion in man is rapidly hydrolysed to release the free amino acids alanine and glutamine (Albers *et al.*, 1988, 1989).

The aim of this present study was to measure directly, using a primed constant infusion of [1-^{13}C]leucine and determination of its incorporation into muscle protein, the acute effects of conventional TPN supplemented by Ala-Gln in patients undergoing major elective upper gastrointestinal surgery. Comparison was

made first with the effect on muscle protein synthesis (in the same patients) of provision of TPN supplemented by isonitrogenous amounts of the free amino acids L-alanine and glycine and secondly with the effects of TPN alone in a different group of patients.

5.3 Methods

5.3.1 Place of Study

All studies were performed in the intensive care unit of Ninewells Hospital and Medical School, Dundee.

5.3.2 Patients

16 patients were studied over a period of 8 h on the third post-operative day: 8 patients according to a longitudinal study protocol comparing the effect of TPN supplemented by L-alanyl-L-glutamine on skeletal muscle mixed protein fractional synthetic rate with the effect on protein synthesis of TPN supplemented with an isonitrogenous solution of L-alanine and glycine (see protocol 3, Chapter 2). The remaining 8 patients were studied as a control group (cross-sectional study) to determine the rates of protein synthesis with provision of TPN alone (see protocol 2, Chapter 2). All patients had localised carcinoma of the lower third of oesophagus and had been admitted electively into one of the general surgical wards for curative

oesophagogastrectomy (Chapter 2). This particular patient group was selected for study because the planned surgery was major and perceived as likely to cause significant perturbation of whole-body protein metabolism. Also, for a variety of clinical reasons (e.g. the need to avoid the oral route post-operatively to protect intestinal anastomotic suture lines in the early post-operative period and post-operative ileus) as well as on nutritional grounds, this group of patients would routinely receive post-operative parenteral nutrition. Details of the patients' age, morphological data and pre-operative plasma albumin and total protein are provided in Table 5.1a. Details of the length of operation, operative blood loss and blood replacement required by transfusion are given in Table 5.1b. All patients were evaluated on admission to ICU to allocate an APACHE II score (Acute Physiology and Chronic Health Evaluation, second modification (Knaus *et al.*, 1985)), thus making a formal assessment of the severity of the acute systemic pathophysiological disturbance resulting from the trauma of surgery, on a background of their long-term health status. The mean APACHE II scores are given in Table 5.1b.

5.3.3 Ethical Approval and Informed Consent

Study protocols were reviewed and approved by the Tayside Ethical Committee for Medical Research. Potentially suitable patients were initially approached on their admission to hospital. During a 2 hour interview, the purpose of the research project and the proposed study details were discussed in detail and patients were also familiarised with the various study instruments and equipment

Table 5.1a Age, morphology and pre-operative nutritional state. CI = 95% confidence intervals.

	Cross-sectional study group			Longitudinal study group		
	Mean ± SEM	Range	CI	Mean ± SEM	Range	CI
Age (y)	67.8 ± 3.4	53-84	(59, 76)	70 ± 1.5	66-79	(66, 74)
Wt (kg)	62.7 ± 4.3	44.5-84	(52, 74)	65.1 ± 5.1	44-85	(52, 78)
Body fat (%)	21 ± 1.2	16.2-26.5	(18, 24)	21.9 ± 1.1	17-26.5	(19, 25)
Fat (kg)	13.2 ± 1.3	9.4-19.7	(10, 16)	14.3 ± 1.4	8.4-20.4	(11, 18)
FFM (%)	79.1 ± 1.2	73.5-83.8	(76, 82)	78.1 ± 1.1	73.5-83	(75, 81)
FFM (kg)	49.5 ± 3.4	33.8-64.3	(41, 58)	50.7 ± 3.9	34.6-64.6	(41, 61)
MAC (cm)	26.9 ± 0.9	22-29.5	(25, 29)	26 ± 0.8	23-29.5	(24, 28)
BMI	22.9 ± 1.1	17.6-27.4	(20, 26)	23.5 ± 1.2	18.3-28.7	(21, 26)
Alb. (g/dl)	41.5 ± 1.4	36-47	(38, 45)	36.4 ± 1.5	31-42	(33, 40)
TP (g/dl)	67 ± 00	60-74	(63, 71)	60.3 ± 2.6	48-70	(54, 66)

MAC = Mid arm circumference
FFM = Fat free mass
BMI = Body mass index

Alb. = Albumin
TP = Total protein
Wt = Weight

Table 5.1b The duration of operation, operative blood loss and post-operative APACHE II score. CI = 95% confidence intervals.

	Cross-sectional study group			Longitudinal study group		
	Mean±SEM	Range	CI	Mean±SEM	Range	CI
Length of operation (min)	263.1±40.9	150-465	(166, 360)	286±34.2	180-415	(205, 367)
Blood loss (ml)	2600±918	250-6600	(429, 4771)	1425±327	500-3200	(652, 2198)
Blood transfused (ml)	1262±557	0-4400	(-54, 2579)	750±245	0-2000	(170, 1330)
APACHE II score*	11.3±1.1	6-15	(8.6-13.9)	9.1±0.6	6 - 11	(7.6, 10.6)

* APACHE II = Acute Physiology and Chronic Health Evaluation score, second modification (Knaus *et al.*, 1985).

used. A written information sheet was also provided to complement the verbal information provided and patients were entered for study after obtaining their written informed consent.

5.3.4 Materials

5.3.4.1 TPN

All patients received parenteral nutrition from the first post-operative day. The nutrition solution consisted of a 50:50 mixture of two commercially available mixed amino acid solutions (Synthamin 9, 500 ml and Synthamin 14, 500 ml, from Baxter Healthcare, Thetford, UK) providing 11.5 g nitrogen (Table 2.3, Chapter 2). In addition, 7532 kJ were provided as 450 g glucose, electrolyte replacement as 70 mmol Na^+ and 80 mmol K^+, all in a total volume of 1.5 l water per 24 h. Patients therefore received a combined total of a 2.5 l bag of TPN solution over 24 h, provided at a constant rate by volumetric infusion pump.

5.3.4.2 Alanine, Glycine and Ala-Gln

Pre-sterilised solutions (prepared in the Department of Pharmacy, Ninewells Hospital and Medical School, Dundee) of the free amino acids L-alanine and glycine (Kabi-Vitrum, Sweden), in 250 ml 0.9% saline (Baxter Healthcare, Thetford, England) were infused *via* a 0.22 μm filter (Millipore SA, Molsheim, France) at

constant rates of 4.8 mg/kg/h and 8.0 mg/kg/h respectively (after priming bolus doses of 4.8 mg/kg and 8.0 mg/kg respectively in a volume of 20 ml 0.9% saline), providing a total of 2.25 mg amino-N/kg/h, for 4 h during the first half of the 8 h tracer infusion study. A sterile solution of the dipeptide L-alanyl-L-glutamine (Ala-Gln, Kabi-Pfrimmer, Germany) in 250 ml 0.9% saline (Baxter Healthcare, Thetford, England) was infused at a constant 11.7 mg/kg/h (after a priming bolus of 11.7 mg/kg in 20 ml 0.9% saline), providing 2.25 mg amino-N/kg/h, for 4 h during the second half of the 8 h tracer infusion study.

5.3.4.3 Stable Isotope Tracers

The stable isotope labelled amino acid L-[1-^{13}C]leucine (99% ^{13}C) and sodium [^{13}C]bicarbonate were purchased from Tracer Technologies Incorporated, Newton, MA, U.S.A. L-[1-^{13}C]leucine was mixed in sterile pyrogen-free sodium chloride (NaCl) solution (Baxter Healthcare, Norfolk, U.K.) immediately prior to administration using an aseptic technique and was sterilised by passage through a 0.22 μm filter (Millipore SA, Molsheim, France). The tracer infusate was subsequently diluted to a total volume of 500 ml in sterile pyrogen-free NaCl solution (Baxter Healthcare, Norfolk, U.K.) and was infused at a constant rate *via* a second 0.22 μm filter at a rate of 1.0 mg/kg/h over 8 h by volumetric pump. L-[1-^{13}C]leucine (1 mg/kg) and sodium [^{13}C]bicarbonate (0.24 mg/kg) used for priming were prepared and administered in similar careful fashion except that the volume of 0.9% NaCl solution used was 20 ml and it was given as a bolus over 3 min.

5.3.5 Placement of Cannulas

A triple lumen cannula was inserted antegradely into the internal jugular vein by the anaesthetist in charge at the time of surgery. This was initially used in the first 24-48 hours to monitor central venous pressure and to administer intravenous medication as required. Cannula patency was maintained by the slow infusion of 0.9% saline (Baxter Healthcare, Norfolk, 500 ml per 24 h). Also, from the first post-operative day, one channel was used to deliver TPN. The remaining channels were used on the day of the metabolic study for constant infusion of labelled amino acids as well as the dipeptide and control solutions.

Deep venous blood was obtained from the "study" forearm from a cannula inserted retrogradely into the median cephalic or basilic ante-cubital vein. Arterial blood was obtained from an intra-arterial cannula sited in the radial artery (at the wrist) for routine post-operative monitoring of mean arterial blood pressure. Blood samples were obtained for determination of plasma amino acid and glucose concentrations, free leucine and α-ketoisocaproate (α-KIC) concentrations and ^{13}C isotope enrichment and hormonal (C-peptide, insulin, glucagon, IGF-I) profiles.

5.3.6 Muscle Biopsies

Three leg muscle (tibialis anterior) biopsies were carried out using conchotome forceps (Chapter 2): i) at the time of surgery for baseline sample for isotope enrichment and free amino acid concentration ii) on the third post-operative day, at the end of the first 4 h period of isotope infusion whilst the patients

received TPN and Ala+Gly and iii) at the end of the second 4 h period of isotope infusion whilst receiving TPN and Ala-Gln infusion. Muscle samples were blotted dry to remove any blood; also, any evident fatty tissue was removed prior to immersion and subsequent storage in liquid nitrogen at -196°C.

5.3.7 Blood and Breath Samples

Blood samples were obtained for amino acid and α-KIC concentrations, α-KIC and ^{13}C-leucine enrichment and hormone profiles (C-peptide, insulin, glucagon and IGF-I). Attempts were made to collect breath samples for CO_2 production rate by Douglas bag and ^{13}C enrichment of expired CO_2 (according to Protocols 2 and 3, Chapter 2) in order to measure leucine oxidation; however most patients were unable to tolerate breath collections so soon after major upper gastrointestinal surgery using a thoracic approach and so collections were abandoned.

5.3.8 Laboratory Analysis

The laboratory analytical techniques used in this study are described in Chapter 2, section 2.17. Additionally, muscle water content was determined for the longitudinal study samples by separating and weighing a portion of each biopsy sample (approximately 10%, by wet weight) then freeze-drying this portion to a constant weight. The change in weight, related to the wet weight, was expressed as percentage muscle water content.

5.4 Results

5.4.1 Arterial Plasma Free Amino and Keto Acid Concentrations

In the longitudinal study, the mean plasma arterial glutamine concentration (Figure 5.1and Table 5.2) was 330±16 (281, 364) μmol/l (mean±SEM, 95% confidence intervals) from the period -30 min to 0 min, falling slightly to 314±16 (274, 355) μmol/l during infusion with Ala+Gly, although this change was not statistically significant; with the infusion of Ala-Gln, the glutamine concentration rose by 29% to 404±30 (316, 471) μmol/l ($p<0.05$). This latter value was also higher than the value of 340±11 (311, 370) μmol/l ($p<0.04$, 1-tailed) for the concentration of glutamine between 90 to 480 min in the cross-sectional study (Table 5.3). In the longitudinal study, the concentration of alanine rose from a basal value of 187±32 (92, 259) μmol/l to a value of 243±26 (173, 306) μmol/l during Ala+Gly infusion ($p<0.03$, 1-tailed) remaining stable at 235±22 (179, 291) μmol/l with Ala-Gln infusion. The concentration of glycine rose by 57% from the basal value with Ala+Gly infusion, from 147.3±11 (103, 158) μmol/l to 231±11 (191, 247) μmol/l, in the longitudinal study, falling back to a near-basal level of 127±7 (105, 142) μmol/l with Ala-Gln infusion. The plasma concentration of leucine was stable during each phase of the longitudinal study (Figure 5.2). The mean leucine concentrations in the longitudinal study for the basal, Ala+Gly and Ala-Gln periods were 123±13 (90, 156) μmol/l, 121±11 (93, 150) μmol/l and 119±7 (102, 136) μmol/l respectively and there were no statistical differences between these values. The corresponding basal value and the value during 90-480 min for the cross-sectional study group were similar at 96±20 (48, 144) μmol/l and 123±9 (101, 146) μmol/l respectively. The

Table 5.2 Arterial plasma amino acid concentrations (longitudinal study). CI = 95% confidence intervals.

Amino acid	Basal		Ala+Gly+TPN (60-240 min)		Ala-Gln+TPN (300-480 min)	
	μmol/l	CI	μmol/l	CI	μmol/l	CI
ALA	187.2 ± 32	(92, 259)	243.1 ± 26	(173, 306)	234.9 ± 22	(179, 291)
ARG	56.1 ± 10	(27, 77)	43.0 ± 6	(23, 56)	42.4 ± 9	(19, 64)
ASP	6.1 ± 1.9	(2, 12)	4.2 ± 1.4	(2, 9)	4.4 ± 1.0	(1.6, 7)
ASN	23.7 ± 5	(9, 35)	21.3 ± 5	(8, 33)	18.6 ± 5	(7, 30)
GLU	40.7 ± 7	(23, 61)	33.5 ± 5	(23, 50)	38.5 ± 7	(19, 56)
GLN	329.5 ± 16	(281, 364)	313.8 ± 16	(274, 355)	404.4 ± 30	(316, 471)
GLY	147.3 ± 11	(103, 158)	231.3 ± 11	(191, 247)	126.9 ± 7	(105, 142)
HIS	51.8 ± 3	(43, 60)	44.5 ± 3	(41, 49)	46.0 ± 4	(33, 58)
3-MH	3.71 ± 0.4	(2.8, 5.1)	3.57 ± 0.3	(3, 4.9)	3.00 ± 0.4	(1.7, 4.1)
ISOLEU	61.3 ± 12	(34, 98)	47.1 ± 9	(29, 75)	55.4 ± 12	(23, 84)
LEU	116.0 ± 10	(70, 121)	106.7 ± 9	(72, 117)	95.2 ± 5	(79, 106)

(*continued on next page*)

Table 5.2 (*continued*)

Amino acid	Basal		Ala+Gly+TPN (60-240 min)		Ala-Gln+TPN (300-480 min)	
	μmol/l	CI	μmol/l	CI	μmol/l	CI
LYS	144.3 ± 16	(93, 176)	134.2 ± 17	(82, 172)	118.0 ± 23	(54, 175)
MET	24.6 ± 4	(15, 33)	20.2 ± 2	(15, 26)	20.0 ± 3	(12, 27)
PHE	97.7 ± 8	(79, 116)	92.9 ± 5	(80, 106)	96.0 ± 6	(82, 110)
SER	84.8 ± 13	(53, 119)	83.1 ± 11	(61, 116)	83.4 ± 12	(50, 113)
TAU	36.2 ± 2	(31, 38)	30.8 ± 1	(28, 33)	29.3 ± 2	(24, 33)
THR	82.5 ± 9	(55, 104)	70.5 ± 6	(52, 86)	66.1 ± 9	(43, 86)
TYR	54.3 ± 7	(34, 71)	48.6 ± 6	(32, 64)	46.3 ± 5	(30, 60)
TRY	31.2 ± 2	(26, 36)	27.0 ± 2	(22, 34)	25.5 ± 3	(18, 32)
VAL	181.5 ± 29	(128, 278)	171.8 ± 30	(118, 269)	191.9 ± 25	(103, 270)
BCAA	358.8 ± 52	(232, 497)	325.7 ± 55	(219, 423)	342.5 ± 60	(179, 487)

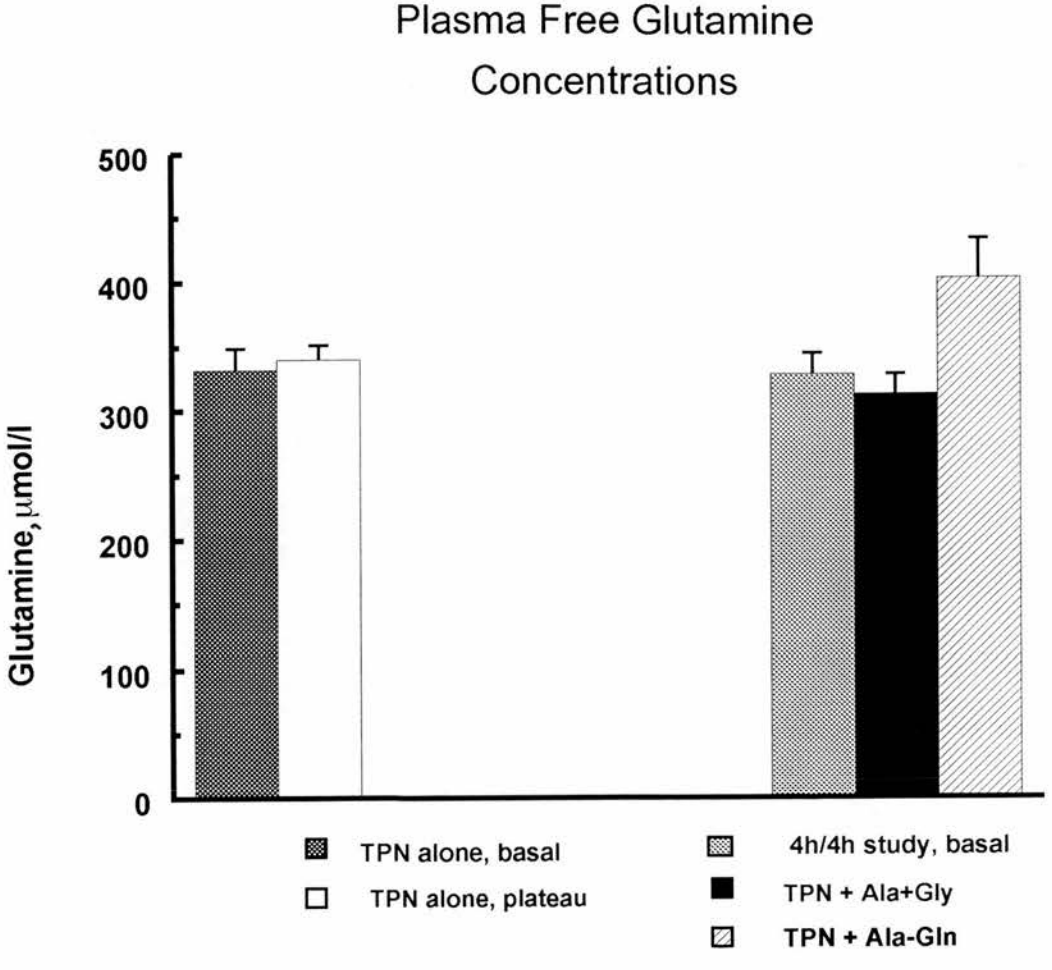

Figure 5.1 Free glutamine concentrations in arterial plasma representing basal (dark grey stippled bar) and plateau (90-480 min, unfilled bar) values for the patient group receiving TPN alone; basal, (light grey stippled bar) and values at plateau for patients receiving TPN supplemented with Ala+Gly (60-240 min, filled bar) and TPN supplemented with Ala-Gln (300-480 min, hatched bar).

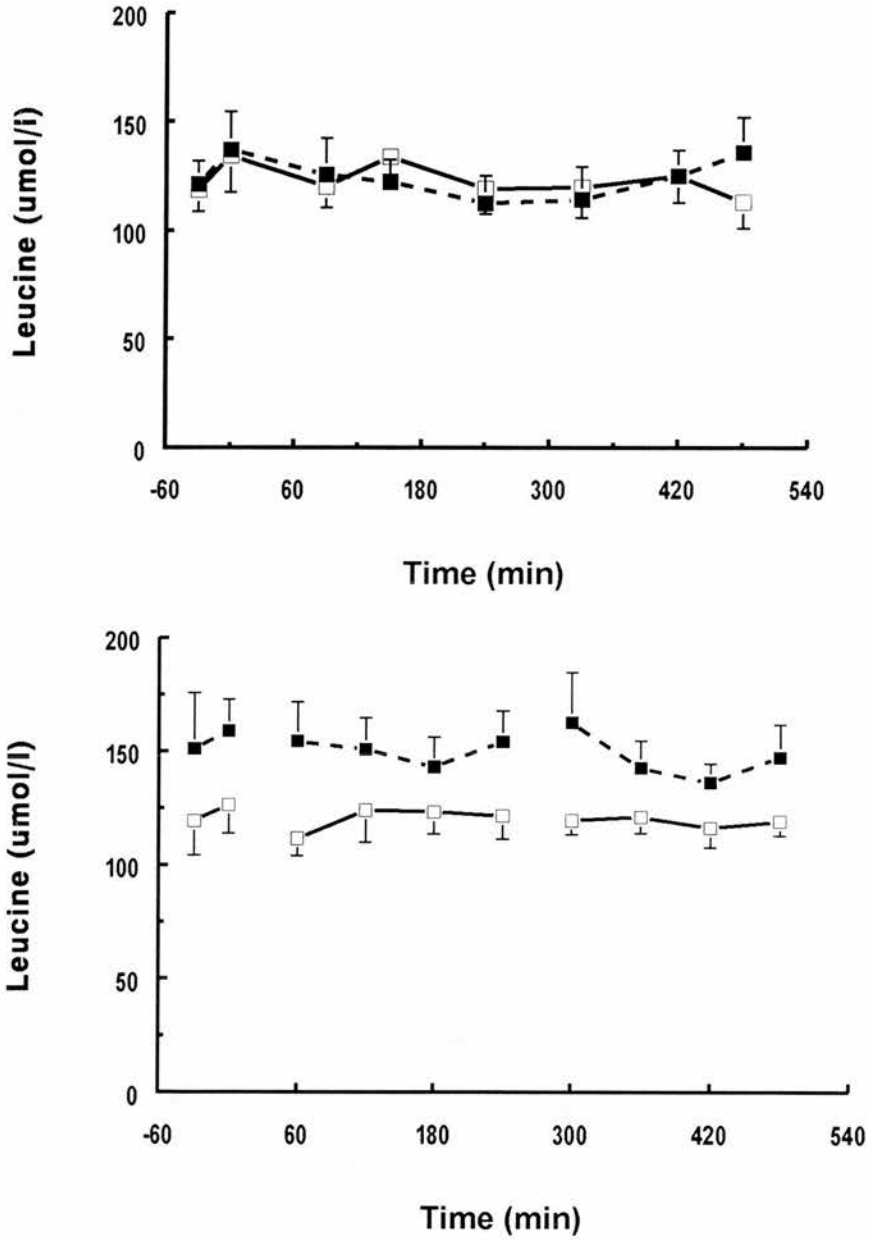

Figure 5.2 Plasma arterial (□) and venous (■) concentrations of free leucine in the patient group receiving TPN alone (90-480 min; top graph) and the group receiving TPN supplemented by Ala+Gly (60-240 min) and Ala-Gln (300-480 min; bottom graph).

Table 5.3 Plasma arterial free amino acid concentrations (cross-sectional study). CI = 95% confidence intervals.

Amino acid	Arterial (basal) μmol/l	CI	Arterial (60-480 min) μmol/l	CI
ALA	174.9 ± 20	(124, 226)	176.8 ± 16	(134, 221)
ARG	51.7 ± 4	(43, 61)	54.5 ± 4	(44, 65)
ASP	3.7 ± 0.6	(2.2, 5.3)	3.7 ± 0.4	(2.6, 4.7)
ASN	32.3 ± 5	(19, 46)	27.7 ± 3	(20, 35)
GLU	40.6 ± 6	(24, 57)	40.2 ± 5	(26, 54)
GLN	331.9 ± 17	(287, 377)	340.4 ± 11	(311, 370)
GLY	189.7 ± 17	(146, 234)	238.7 ± 34	(148, 329)
HIS	59.5 ± 3	(51, 68)	65.8 ± 6	(49, 83)
3-MH	3.80 ± 0.6	(2.4, 5.4)	3.75 ± 0.6	(2.2, 5.4)
ISOLEU	57.8 ± 7	(40, 76)	54.8 ± 7	(35, 74)
LEU	95.6 ± 20	(48, 144)	123.4 ± 9	(101, 145)
LYS	157.7 ± 11	(130, 186)	147.9 ± 13	(113, 183)
MET	39.2 ± 8	(18, 60)	29.3 ± 3	(22, 37)
PHE	118.3 ± 9	(98, 139)	115.5 ± 8	(97, 134)
SER	78.1 ± 5	(66, 91)	79 ± 6	(63, 95)
TAU	36.3 ± 2	(31, 42)	29.8 ± 2	(26, 34)
THR	87.1 ± 7	(70, 105)	90.9 ± 6	(75, 106)
TYR	66.5 ± 4	(57, 76)	65.1 ± 4	(55, 75)
TRY	36.8 ± 4	(26, 48)	43.1 ± 7	(24, 62)
VAL	175.6 ± 13	(140, 211)	179.7 ± 8	(158, 201)
BCAA	329.0 ± 23	(269, 388)	357.9 ± 24	(295, 421)

concentration of 3-methylhistidine was lower during TPN + Ala-Gln infusion than during TPN + Ala+Gly, the respective values being 3.00±0.4 (1.7, 4.1) μmol/l and 3.57±0.3 (3.0, 4.9) μmol/l.

5.4.2.1 Intramuscular Water Content

Data was available for the longitudinal study only (Figure 5.3). The pre-operative muscle water content was 69.9±2 (66, 74) %, rising to 76.8±0.7 (75, 78) % with TPN + Ala+Gly and remaining at the same value at 76.8±0.4 (76, 78) % with TPN + Ala-Gln infusion.

5.4.2.2 Intramuscular Free Amino Acid Concentrations

The intramuscular concentration of free amino acids in the two groups in the pre- and both post-operative muscle biopsy samples are shown in Tables 5.4a and 5.5. In the longitudinal study, the intramuscular concentration of glutamine fell by 51% from the pre-operative value of 62±4 (51, 72) μmol/g protein to the post-operative value of 30±3 (24, 37) μmol/g protein at the end of the Ala+Gly infusion period ($p<0.0005$), rising by a small (13%) but significant amount to 34±3 (26, 42) μmol/g protein at the end of the Ala-Gln infusion ($p<0.03$, 1-tailed) (Table 5.4b and Figure 5.4), although this value was still 45% lower than the pre-operative value. The pre-operative and end-of-study post-operative intramuscular glutamine concentrations in the cross-sectional study group were 60±5 (48, 72) and 28±4 (19, 37) μmol/g protein (falling by 53%), similar to the corresponding pre-operative and M2 (end of Ala+Gly infusion) values in the longitudinal study. In the longitudinal study, there were falls in the concentrations of arginine, histidine, lysine and tyrosine from pre-operative values and corresponding rises in concentrations of the amino acids phenylalanine and tryptophan. There were no significant differences

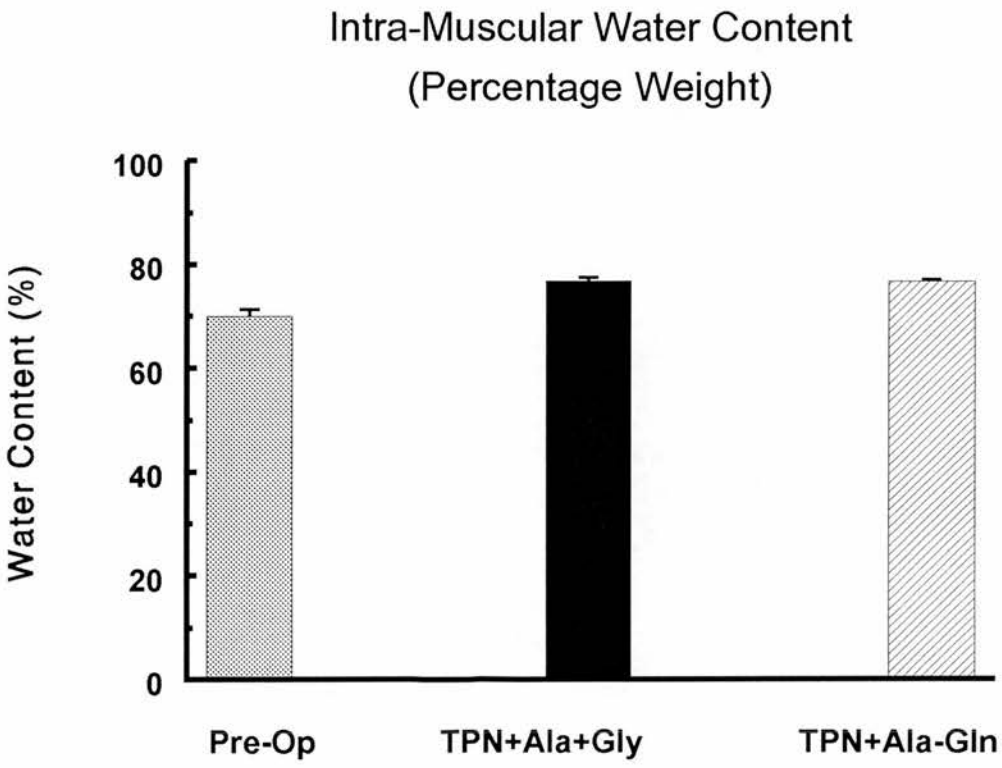

Figure 5.3 Intramuscular water content, expressed as a percentage of initial muscle sample wet weight, in the longitudinal study group: pre-operative sample, stippled bar; sample at end of Ala+Gly supplementation of TPN, filled bar; sample at end of Ala-Gln supplementation of TPN, hatched bar.

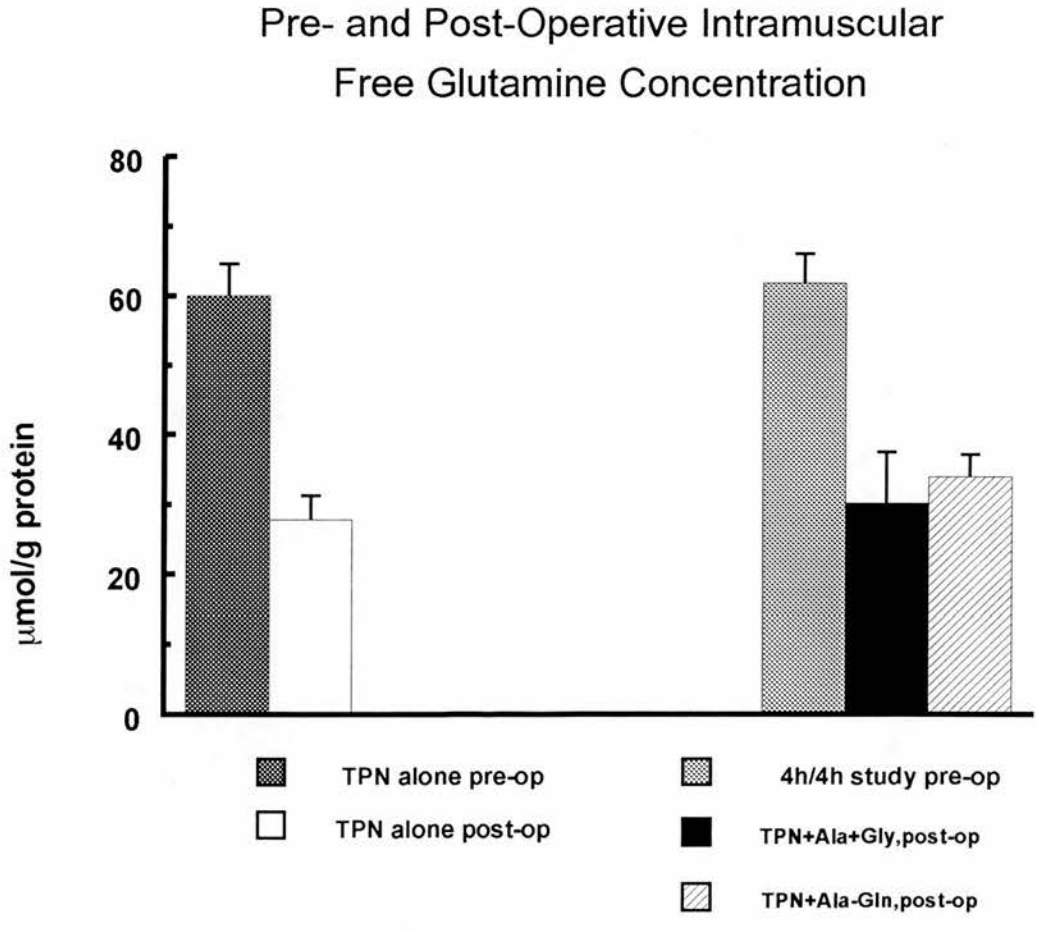

Figure 5.4 Intramuscular free glutamine concentrations. Represented on the left are pre-operative (dark grey stippled bar) and post-operative values (at 480 min, unfilled bar) for the patient group receiving TPN alone; on the right are pre-operative (light grey stippled bar), end of period of TPN + Ala+Gly (at 240 min, filled bar) and TPN + Ala-Gln (at 480 min, hatched bar) values in the longitudinal study group.

in the concentration of BCAA's between the Ala+Gly period and Ala-Gln period.

5.4.3 Plasma Hormones and Substrates

The concentrations of plasma C-peptide, insulin, glucagon, IGF-I and of blood D-(-)-3-hydroxybutyrate were stable within each study period in the split period study (Table 5.6). Neither supplementation of TPN by the Ala+Gly mixture nor by Ala-Gln dipeptide significantly altered the concentrations of these hormones and metabolites from their pre-study basal values on TPN alone. The basal plasma glucose concentration, in the cross-sectional study group was 8.3±0.4 (7.3, 9.2) mmol/l, and remained unchanged at 8.5±0.7 (6.9, 10.0) mmol/l during the period 90-480 min. The basal value in the longitudinal study was 9.5 (7.6, 11.4) mmol/l, and did not change significantly between the TPN + Ala+Gly (9.0±0.7 (7.2, 10.7) mmol/l) and TPN + Ala-Gln (8.8±0.6 (7.5, 10.2) mmol/l) periods.

5.4.4 Plasma and Muscle Free Leucine Pool Enrichments

For both plasma leucine and α-ketoisocaproate, plateau enrichment was attained during the last 180 min of each phase of the longitudinal study and during the last 420 min of the cross-sectional study (Figures 5.5 and 5.6 and Table 5.7). Also, the plasma concentration of α-ketoisocaproate was stable during each phase of the longitudinal study (Figure 5.7) and plateau concentrations were attained and maintained in the period 60-480 min of the cross-sectional study (Figure 5.7). In longitudinal study, there was a rise in plasma leucine enrichment

Table 5.4a Intramuscular free amino acid concentrations (μmol/g protein) (Longitudinal study group). CI = 95% confidence intervals.

Amino acid	Pre-op (M1)		Post-op (M2)		Post-op (M3)	
	μmol/g	CI	μmol/g	CI	μmol/g	CI
ALA	12.4 ± 1.4	(8.9, 15.8)	11.7 ± 0.8	(9.8, 13.6)	12.1 ± 1.1	(9.1, 14.9)
ARG	4.0 ± 0.5	(2.7, 5.2)	1.0 ± 0.2	(0.5, 1.5)	1.0 ± 0.2	(0.5, 1.6)
ASP	3.9 ± 0.6	(2.4, 5.4)	3.1 ± 0.3	(2.4, 3.8)	4.3 ± 0.7	(2.6, 6)
GLU	11.6 ± 1.6	(7.6, 15.6)	12.9 ± 1.5	(9.2, 16.6)	13.1 ± 2	(8, 18.2)
GLN	61.7 ± 4.2	(51.1, 72.4)	30.2 ± 2.6	(23.7, 36.7)	33.9 ± 3.2	(25.8, 42.1)
GLY	6.7 ± 0.5	(5.3, 8)	6.1 ± 0.5	(4.8, 7.4)	5.8 ± 0.4	(4.8, 6.8)
HIS	1.5 ± 0.1	(1.2, 1.7)	0.9 ± 0.1	(0.6, 1.1)	1.1 ± 0.2	(0.5, 1.1)
ISO	0.3 ± 0.0	(0.2, 0.4)	0.5 ± 0.1	(0.2, 0.8)	0.5 ± 0.1	(0.2, 0.7)
LEU	0.9 ± 0.1	(0.6, 1.1)	1.2 ± 0.2	(0.6, 1.8)	1.1 ± 0.2	(0.7, 1.6)
LYS	10.4 ± 2.5	(4.1, 16.7)	2.9 ± 1.5	(-0.9, 6.7)	1.5 ± 0.7	(-0.4, 5.2)
PHE	0.4 ± 0.0	(0.3, 0.5)	0.8 ± 0.1	(0.6, 0.9)	0.7 ± 0.1	(0.6, 0.9)
SER	3.0 ± 0.4	(1.9, 4.1)	3 ± 0.3	(2.2, 3.7)	3 ± 0.2	(2.5, 3.5)
TAU	33.6 ± 2.4	(27.5, 39.7)	84.7 ± 5	(72.2, 97.3)	93.1 ± 6	(76.3, 110.3)
THR	2.9 ± 0.2	(2.3, 3.5)	2.4 ± 0.4	(1.5, 3.3)	2.7 ± 0.3	(2, 3.4)
TRY	18 ± 3.7	(8.6, 27.4)	29.3 ± 3.4	(20.7, 38)	31.7 ± 3	(24, 39.4)
TYR	0.4 ± 0.1	(0.3, 0.5)	0.6 ± 0.1	(0.4, 0.8)	0.6 ± 0	(0.5, 0.7)
VAL	1.7 ± 0.1	(1.4, 2)	1.8. ± 0.2	(1.3, 2.2)	1.8 ± 0.1	(1.3, 2.1)
BCAA	2.9 ± 0.3	2.3, 3.6)	3.4 ± 0.5	(2.1, 4.7)	3.4 ± 0.4	(2.4, 4.3)

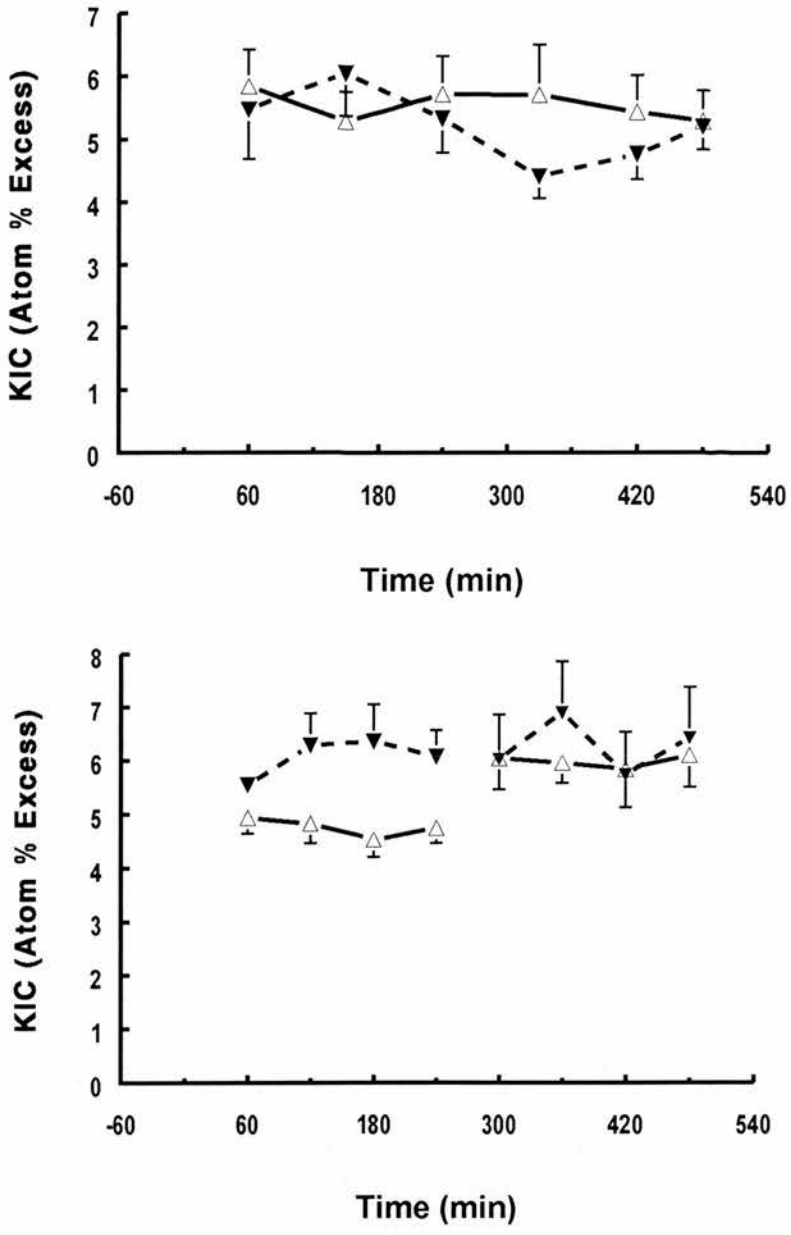

Figure 5.5 Plasma arterial (Δ) and venous (▾) enrichment of α-ketoisocaproate in the group of patients receiving TPN alone (90-480 min, top graph) and the patients receiving TPN supplemented by Ala+Gly (0-240 min) and Ala-Gln (240-480 min; bottom graph).

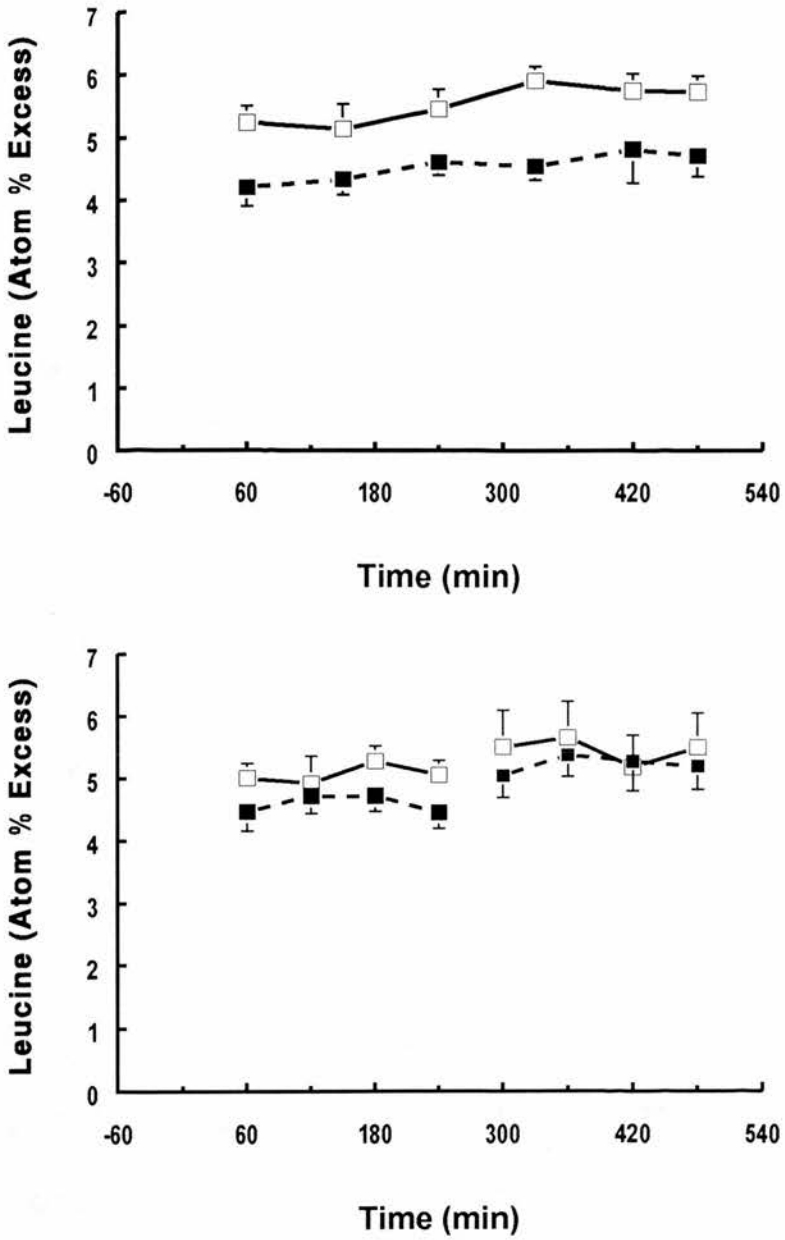

Figure 5.6 Plasma arterial (□) and venous (■) enrichment of free leucine in the patient group receiving TPN alone (90-480 min; top graph) and the group receiving TPN supplemented by Ala+Gly (0-240 min) and Ala-Gln (240-480 min; bottom graph).

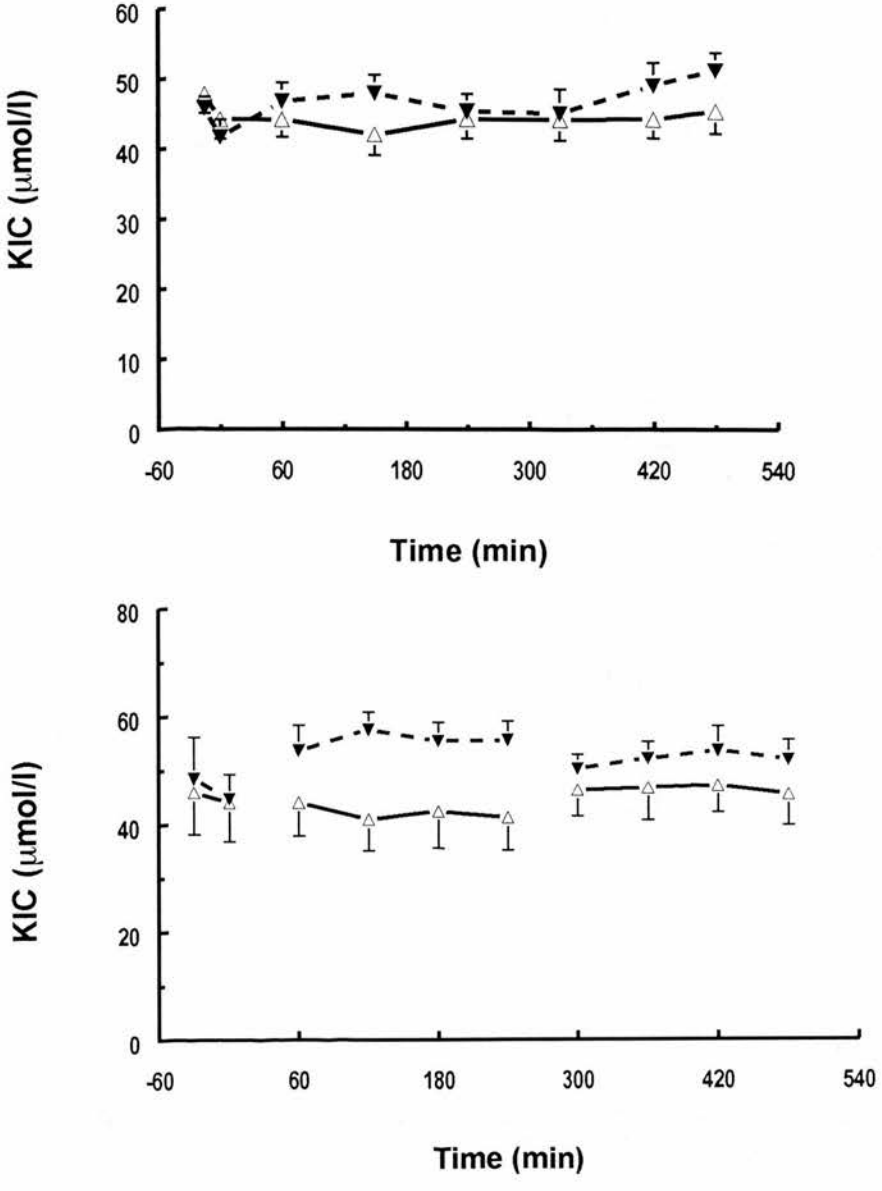

Figure 5.7 Plasma arterial (Δ) and venous (▾) concentrations of α-ketoisocaproate in the patient group receiving TPN alone (90-480 min; top graph) and the group receiving TPN supplemented by Ala+Gly (60-240 min) and Ala-Gln (300-480 min; bottom graph).

Table 5.4b Intramuscular free glutamine concentrations (μmol/g protein; individual patients' values: longitudinal study). CI = 95% confidence intervals.

Subject	Pre-op (M1) μmol/g	Post-op (M2) μmol/g	Post-op (M2) μmol/g
1	54.8	30.7	31.4
2	75.2	37.5	50.1
3	46.6	29.0	28.5
4	55.4	20.0	22.8
5	71.8	25.4	25.4
6	49.6	27.9	31.6
7	81.1	25.9	35.2
8	58.9	45.0	46.7
Mean±SEM	61.7±4.2	30.2±2.6	33.9±3.2
CI	(51.1, 72.4)	(23.7, 36.7)	(25.8, 42.1)

from 5.05±0.2 (4.4, 5.7) APE to 5.49±0.6 (4.0, 7.0) APE and there was also a rise in plasma α-ketoisocaproate enrichment from 4.75±0.3 (4.0, 5.5) APE during Ala+Gly infusion to 6.11±0.6 (4.7, 7.5) APE during Ala-Gln infusion. Neither change reached statistical significance (p=0.30 and p=0.066, respectively). The intracellular leucine enrichment remained unchanged (3.21±0.2 (2.8, 3.6) *versus* 3.24±0.1 (3.0, 3.5) APE). The ^{13}C intracellular leucine enrichment was 63% of that of plasma leucine during Ala+Gly infusion, remaining at a similar value of 59% during Ala-Gln infusion. The ^{13}C intracellular leucine enrichment was 68% of that of plasma α-ketoisocaproate during Ala+Gly infusion, falling to 53% during Ala-Gln

infusion.

5.4.5 Muscle Protein Synthesis

In the longitudinal study, the fractional rate of protein synthesis in T. anterior muscle during Ala+Gly infusion, calculated on the basis that the mean ^{13}C enrichment in plasma α-ketoisocaproate (Table 5.7) should most closely the precursor synthetic pool of *t*RNA and provide the best estimate of protein synthesis, was 0.025±0.003 (0.016, 0.034) %/h; the rate increased during Ala-Gln infusion by 88% to 0.047±0.006 (0.031, 0.062) %/h (p=0.0052) (Figures 5.8 and 5.9). The fractional rate of protein synthesis with TPN infusion alone, also using α-ketoisocaproate as the 'surrogate' precursor, was 0.031± 0.003 (0.026, 0.036) %/h, not significantly different from the rate observed in the longitudinal study during Ala+Gly infusion but significantly lower than the rate observed during the Ala-Gln period ($p<0.05$). These values may be considered as the best estimates of protein synthesis. For the longitudinal study, when calculating the protein synthetic rate on the basis of the ^{13}C enrichment of the muscle intracellular free leucine pool, a value (the probable upper limit) of 0.039±0.003 (0.032, 0.047) was obtained during the Ala+Gly infusion period, rising by 92% to 0.075±0.008 (0.055. 0.096) %/h (p=0.0095) during Ala-Gln infusion. The rate of protein synthesis in the cross-sectional group, calculated on the same basis, was 0.049±0.005 %/h (0.037, 0.061), and this value was not significantly different to the corresponding value in the longitudinal study group during the Ala+Gly infusion, but significantly slower than that obtained during Ala-Gln infusion (p=0.04).

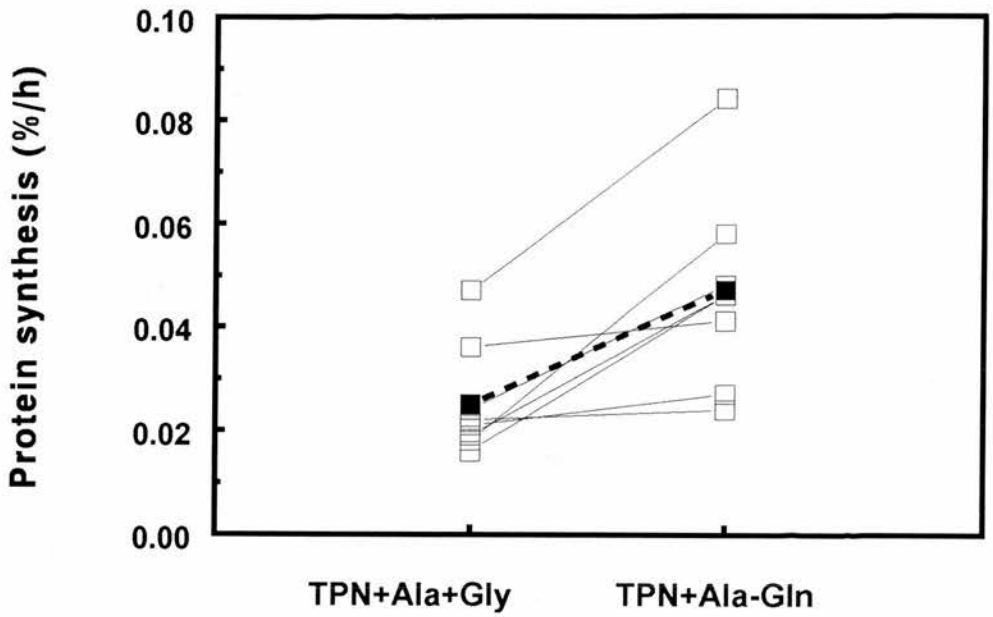

Figure 5.8 Fractional muscle protein synthesis rate by [1-^{13}C]leucine incorporation, calculated using plasma α-ketoisocaproate as the precursor pool, in the group receiving TPN supplemented by Ala+Gly (on the left) and Ala-Gln (on the right). The hatched bar connects the points representing the mean synthetic rates for each period.

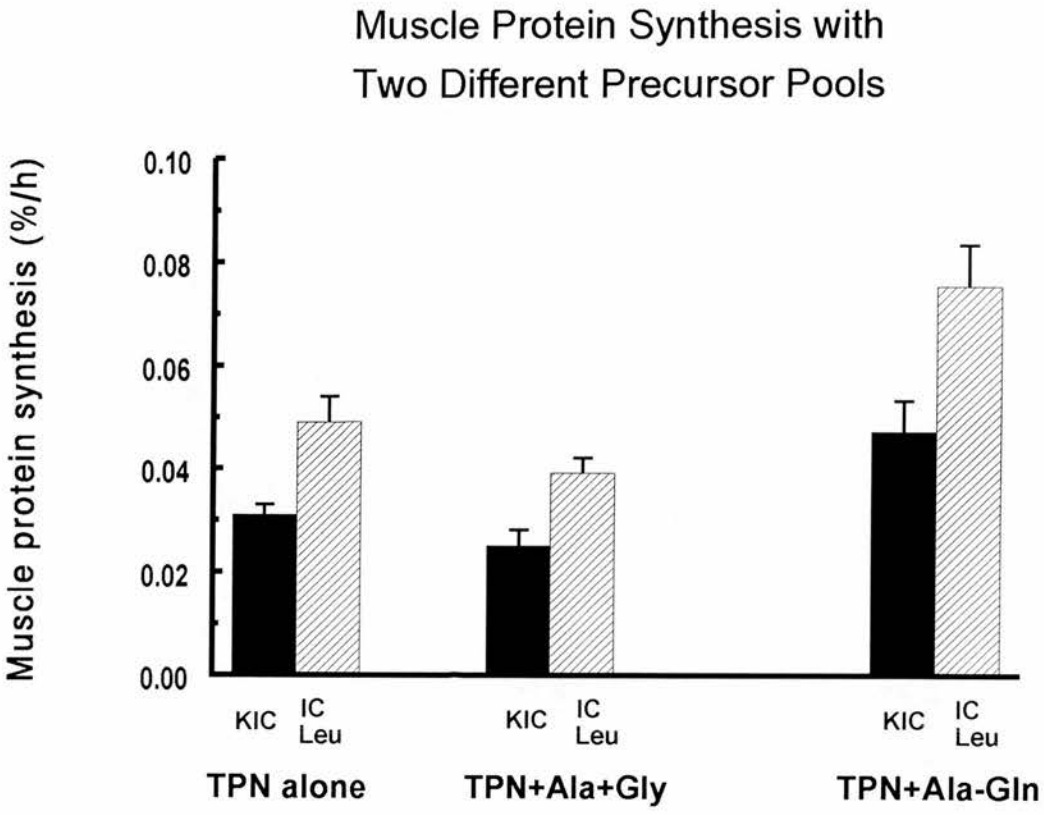

Figure 5.9 Skeletal muscle fractional mixed protein synthesis, calculated separately using the surrogate precursor pools (for the true precursor, *t*RNA) for protein synthesis, of α-ketoisocaproate (unfilled bar) and intracellular (intramuscular) free leucine (hatched bar), in the patient group receiving TPN alone and the group receiving TPN supplemented by Ala+Gly and Ala-Gln.

Table 5.5 Free intramuscular amino acid concentrations (μmol/g protein; cross-sectional study).

Amino acid	Pre-Op (M1) μmol/g	CI	Post-op (M2) μmol/g	CI
ALA	10.8 $\pm$ 0.7	(9.0, 12.5)	12.6 $\pm$ 0.4	(11.5, 13.6)
ARG	5 $\pm$ 0.5	(3.8, 6.3)	1.5 $\pm$ 0.4	(0.6, 2.5)
ASP	5.1 $\pm$ 0.6	(3.5, 6.8)	4.2 $\pm$ 0.3	(3.4, 4.9)
GLU	10.5 $\pm$ 0.9	(8.2, 12.8)	13.2 $\pm$ 1	(10.7, 15.7)
GLN	60 $\pm$ 4.6	(47.8, 72.3)	27.8 $\pm$ 3.5	(18.6, 36.9)
GLY	6.7 $\pm$ 0.4	(5.6, 7.7)	6.6 $\pm$ 0.5	(5.2, 8.0)
HIS	1.6 $\pm$ 0.2	(1.2, 2.1)	0.9 $\pm$ 0.0	(0.8, 1.0)
ISO	0.3 $\pm$ 0.0	(0.2, 0.4)	0.3 $\pm$ 0.1	(0.2, 0.5)
LEU	0.9 $\pm$ 0.1	(0.6, 1.3)	0.8 $\pm$ 0.1	(0.6, 0.9)
LYS	16.3 $\pm$ 2.8	(8.4, 24.1)	3.2 $\pm$ 0.8	(1.0, 5.4)
PHE	0.4 $\pm$ 0.0	(0.2, 0.7)	0.7 $\pm$ 0.1	(0.6, 0.8)
SER	2.2 $\pm$ 0.2	(1.8, 2.6)	2.9 $\pm$ 0.2	(2.4, 3.4)
TAU	27.1 $\pm$ 3.5	(17.8, 36.4)	81.7 $\pm$ 3.9	(70.7, 92.7)
THR	3.1 $\pm$ 0.2	(2.6, 3.5)	2.9 $\pm$ 0.2	(2.3, 3.4)
TRY	33.9 $\pm$ 5.2	(20.7, 47.0)	40.2 $\pm$ 4.4	(29.2, 51.2)
TYR	0.5 $\pm$ 0.0	(0.4, 0.6)	0.5 $\pm$ 0.1	(0.4, 0.6)
VAL	2 $\pm$ 0.2	(1.4, 2.6)	1.6 $\pm$ 0.1	(1.2, 2.0)
BCAA	3.2 $\pm$ 0.3	(2.5, 3.9)	2.6 $\pm$ 0.3	(1.7, 3.5)

5.4.6 Whole-body Leucine Kinetics

Although plasma leucine flux was lower during TPN + Ala-Gln infusion than during TPN + Ala+Gly infusion i.e. 128.7$\pm$4 (118, 140) μmol/kg/h *versus* 141.7$\pm$8 (123, 161) μmol/kg/h, the difference did not reach statistical significance at the 5% level (Figure 5.10). Similarly, the rate of appearance of endogenous leucine, a measure of protein breakdown, was lower, but not by a significant amount, during Ala-Gln infusion, 102.3$\pm$5 (91, 114) μmol/kg/h *versus* 115.3$\pm$6 (101,

Table 5.6 Plasma arterial hormone and metabolite concentrations during the periods TPN + Ala+Gly (60-240 min) and TPN + Ala-Gln, (300-480 min).

Hormone	Pre-op	Basal	CI	TPN+Ala+Gly	CI	TPN+Ala-Gln	CI
C-peptide (nmol/l)	0.75±0.07	1.51±0.20	(1.00, 2.03)	1.73±0.12	(1.48, 1.99)	1.61±0.12	(1.35, 1.88)
Insulin (mU/l)	8.0±2.1	26.7±4.5	(15.4, 38.1)	29.4±2.3	(24.3, 34.5)	24.6±2.6	(18.8, 30.4)
IGF-1 (μg/l)	150.1±28.4	97.0±11.2	(68.6, 125.4)	95.8±6.2	(74.4, 117.1)	93.4±5.0	(76.0, 110.9)
Glucagon (μg/l)	0.09±0.01	0.17±0.02	(0.10, 0.23)	0.2±0.02	(0.13, 0.28)	0.24±0.04	(0.09, 0.39)
B-hydroxy-butyrate (μmol/l)	--	13.4±5.3	(-7.5, 34.3)	29.6±12.2	(-11.0, 70.3)	15.2±4.0	(-0.50, 30.9)

Table 5.7 ^{13}C Enrichment of free leucine and α-ketoisocaproate (α-KIC) in arterial plasma and of free leucine in muscle.

	TPN alone (60-480 min)	CI	TPN + Ala+Gly (60-240 min)	CI	TPN + Ala-Gln (300-480 min)	CI
Plasma leucine (APE)	5.43±0.26	(4.78, 6.09)	5.05±0.24	(4.37, 5.74)	5.49±0.56	(4.00, 6.97)
Plasma α-KIC (APE)	5.48±0.45	(4.22, 6.75)	4.75±0.27	(3.97, 5.52)	6.11±0.58	(4.70, 7.52)
Muscle free leucine (APE)	2.83±0.15	(2.46, 3.21)	3.21±0.17	(2.81, 3.60)	3.24±0.11	(2.98, 3.50)

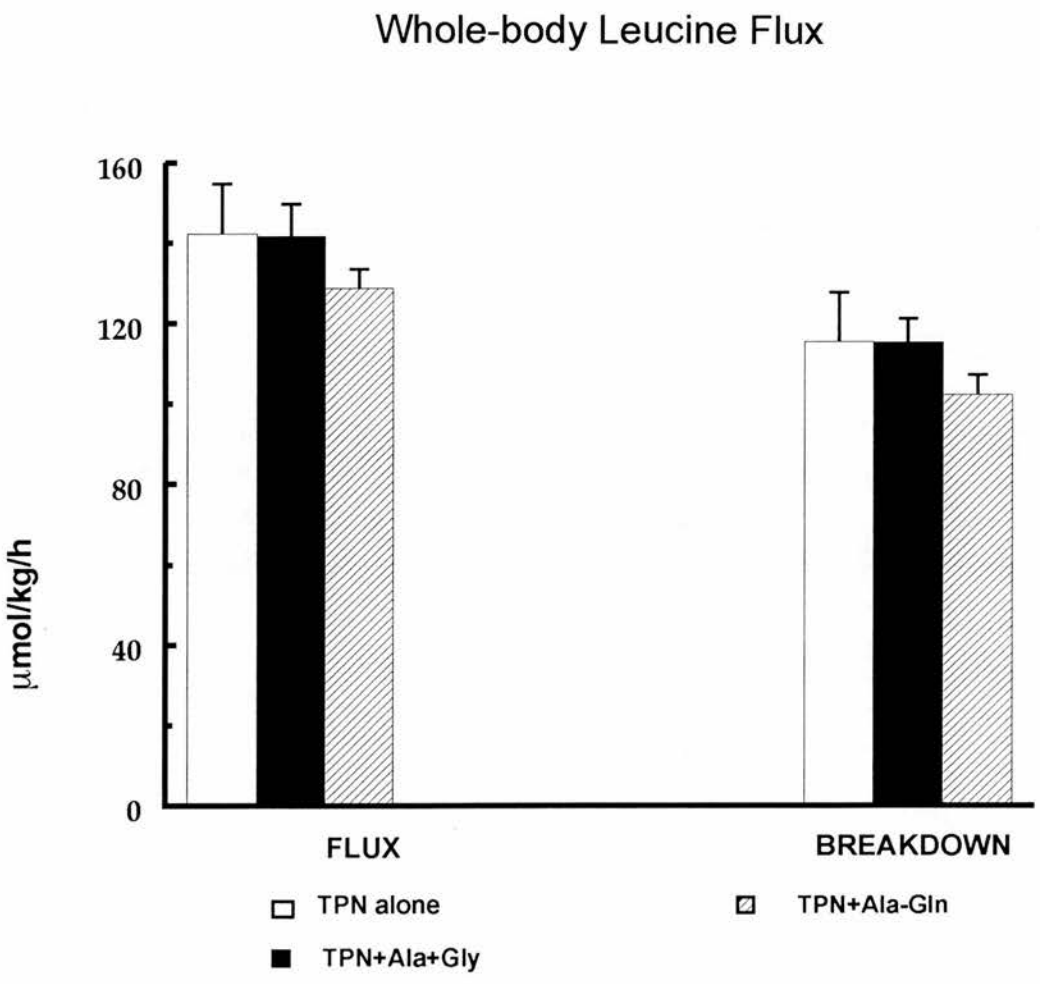

Figure 5.10 Whole-body leucine flux and breakdown for the patient group receiving TPN alone (unfilled bar) and the group receiving TPN supplemented by Ala+Gly (filled bar) and Ala-Gln (hatched bar).

129) μmol/kg/h. The rates of plasma leucine flux and whole-body protein breakdown with TPN alone were 142±13 (113, 172) and 115±12 (86, 144) μmol/kg/h, respectively, these values being identical to the values observed during the Ala+Gly infusion period of the longitudinal study.

5.5 Discussion

As all the clinical studies of post-operative patients reported here were carried out in the ICU, the study environment was entirely different to that in which the volunteer studies were carried out. There were many expected interruptions during the course of each study with patients requiring the attentions of attending nursing and medical staff as well as ancillary staff such as physiotherapists and phlebotomists and it was sometimes difficult to stick rigidly to the blood sampling times as per protocol. Nevertheless, where sampling times differed from intended times, actual times were carefully recorded and taken into account in subsequent calculations.

The laboratory analytical techniques used in this study are well established (Bennet *et al*, 1989) and were felt to be appropriate. Previously established analysis protocols were strictly adhered to in order to minimise laboratory error.

In designing these studies it was considered important to have an isocaloric, isonitrogenous control amino acid solution against which to compare the effects of Ala-Gln supplementation of TPN on protein turnover in patients and the initial rationale for the use of amino acids alanine and glycine was that this mixture had previously been reported to have been safely and effectively

used as a control solution in post-operative patients for studies of Ala-Gln supplementation on nitrogen balance (Stehle *et al.*, 1989). Also, prior to its use in patients, it was used without any side-effects in our studies on the effects of Ala-Gln on muscle protein synthesis and breakdown in healthy subjects (Chapters 3 and 4). Alanine was effectively given in the same amounts during each of the four hour periods, assuming near-complete hydrolysis of Ala-Gln (Albers *et al.*, 1988). Therefore glycine was effectively the control amino acid against which glutamine was compared. To our knowledge there is no evidence that glycine has any significant effect on muscle protein turnover. Indeed, in the rat, no correlation was found between intramuscular glycine concentration and muscle protein synthesis (Jepson *et al.*, 1988); however, as this may not necessarily apply in man, the safeguard used in the present studies was to incorporate into the protocol design a study group receiving TPN alone, for which, if the assumption that glycine has a neutral effect on protein turnover is correct, the synthesis and breakdown rates should not be significantly different from that observed during alanine and glycine supplementation of TPN. This was indeed observed and although the protein synthetic rates were generally a little higher with TPN alone than during TPN supplemented by alanine and glycine, the differences were not significant.

The calculations of muscle protein synthesis are based upon an assumption of attainment of steady state. A time period of 1-1.5 hours is usually required for plasma α-KIC ^{13}C enrichment to reach plateau values during tracer infusion (see Figure 1.5, Chapter 1). Four hours should theoretically, therefore, be an adequate time period for each part of the longitudinal study and this appears to have been the case (see Figure 5.4), since α-KIC enrichment and

concentrations attained, and were maintained, at plateau during the measurement periods 90-240 min and 330 to 480 min of each 4 h study period. Leucine ^{13}C enrichment and leucine concentration similarly reached plateau and were maintained thus over the same time intervals. A similar 4 h / 4 h longitudinal study design was successfully used by Bennet and colleagues to demonstrate the effects of insulin with mixed amino acid infusion on skeletal muscle protein synthesis in both healthy subjects (Bennet *et al.*, 1989) and also in insulin dependant diabetic patients (Bennet *et al.*, 1990).

Nevertheless there were potentially conflicting factors which required consideration prior to adopting the 4 h/ 4 h longitudinal study design in investigating post-surgical patients. Although an ideal protocol may have incorporated longer study periods, e.g. 8 h or 6 h rather than 4 h, a single group of relatively unwell patients would have been unlikely to be able to tolerate 2 such studies 'back-to-back'. However, the use of 2 *different* patient groups introduces additional variables i.e. surgery and anaesthesia (Rennie and MacLennan, 1985; Carli *et al.*, 1989; Essén, 1991; Essén *et al.*, 1992a), post-operative analgesia (Kehlet, 1984), post-operative TPN (Moller-Loswick *et al.*, 1991; Carli *et al.*, 1990; Essén *et al.*, 1993). The potential heterogeneity in the pathophysiological responses to such intervention could possibly make it harder to determine the additional effect of a further intervention i.e. glutamine provision, without the use of larger patient numbers in each group.

As an alternative to the primed constant infusion technique, Garlick and colleagues have applied the 'flooding dose' technique, (Garlick *et al.*, 1989), to measure rates of protein synthesis, by tracer incorporation, in healthy subjects to investigate the effect of feeding (McNurlan *et al.*, 1993) and insulin (McNurlan

et al., 1992) and in patients to determine the effects of anaesthesia (Essén *et al.*, 1992), surgery (Essén, 1991) and post-operative TPN (Essén *et al.*, 1993). The technique aims to flood all rapidly exchanging leucine pools to the same level of labelling with tracer, by administration of a large dose of leucine (0.05 g/kg body weight), of a relatively low level of labelling (20%) over a period of a few minutes. Muscle is sampled after 90 min by biopsy and analysed by the usual methods. The enrichment is related to the average of the tracer-labelling of venous blood α-KIC. Although the flooding dose method has the obvious attraction when applied in patient studies in requiring less time for completion for each individual study, there is now good evidence that this method may itself stimulate muscle protein synthesis (Smith *et al.*, 1992, 1993) and so may be unable to detect additional or further effects of other interventions of interest.

The post-operative falls in intramuscular free glutamine of 51% and 53% in the two groups in this study are almost identical in extent to those previously reported following surgical injury (Vinnars *et al.*, 1975; Carli *et al.*, 1990). Recently, Hammarqvist and colleagues (Hammarqvist *et al.*, 1989) reported a fall in intramuscular free glutamine of 39% in patients undergoing elective cholecystectomy. This lower value may reflect the relatively minor nature of surgery undertaken and consequently the metabolic response was likely to have been less severe than after oesophagogastrectomy, although no formal assessment (by 'injury score') of severity of surgical injury is reported in Hammarqvist's study. In the same study, Hammarqvist and colleagues compared the intramuscular glutamine concentration in the patients receiving conventional TPN with that in another group receiving glutamine-enriched TPN. The 17% observed *attenuation* in the extent of the post-operative intramuscular

glutamine concentration fall (Hammarqvist *et al.*, 1989) is of a similar order to the 13% *rise* in intramuscular glutamine with short-term Ala-Gln supplementation in our study, achieved in association with a 14% rise in plasma glutamine concentration. It is surprising that in Hammarqvist's study, the attenuation of the post-operative muscle free glutamine concentration fall was achieved inspite of a *reduction* in the post-operative plasma free glutamine concentration with glutamine provision; yet interestingly, the control group receiving conventional TPN showed no significant change in their post-operative plasma glutamine concentration.

It did not prove possible in these studies to determine pre-operative values for rates of muscle protein synthesis against which to compare the effect of surgery. A number of studies have now been reported from our centre in the use of the percutaneous conchotome biopsy technique of the muscle tibialis anterior in the investigation of muscle protein synthesis under a variety of circumstances. Bennet and colleagues (Bennet *et al.*, 1989) reported a mean protein synthetic rate in tibialis anterior of 0.055 %/h in healthy young post-absorptive men, rising to 0.074 %/h with a mixed amino acid infusion of Synthamin (Baxter Healthcare, Norfolk, UK). More recent reported work from the same centre, using similar methodology, indicates a slightly lower mean value for tibialis anterior protein synthetic rate of 0.045 %/h in healthy post-absorptive man (Smith *et al.*, 1992). The post-operative rates for protein synthesis in the same muscle, in the present study, were 31-44% lower in post-operative patients inspite of receiving nutrition, i.e., intravenous amino acids in TPN with Ala+Gly or in TPN alone. The higher mean value of 0.047 %h with Ala-Gln supplementation was very similar to the value for post-absorptive man (Smith *et al.*, 1992), mid-way

between the values observed in healthy post-absorptive man receiving Ala+Gly alone (0.042 %/h) or receiving Ala-Gln alone (0.051 %/h) (Chapter 3), and 36% lower than post-absorptive subjects receiving mixed amino acids (0.074 %/h, Bennet *et al.*, 1989). However comparisons such as these can only be made with caution and no firm conclusions can be drawn as the characteristics of the subjects were different (healthy *versus* elderly cancer patients) and the study environments were entirely different.

Although it was my original intention to investigate the effects of Ala-Gln supplementation on whole-body protein turnover by determining whole-body leucine kinetics, a major component of this, whole-body leucine oxidation, could not be measured or calculated as the great majority of patients were unable to tolerate the use of a Douglas bag so soon after their major surgery (particularly as a thoraco-abdominal incision was often used). No portable ventilated hood equipment was available during the time period of these studies and thus the opportunity to investigate this particular aspect of the effect of Ala-Gln could not be taken.

From these findings, the low post-operative intramuscular glutamine concentration does appear to be associated with a reduced muscle protein synthetic rate. Provision of TPN alone or provision of TPN supplemented by a control isonitrogenous solution of Ala+Gly does not raise intramuscular free glutamine nor does it increase the muscle protein synthetic rate. However Ala-Gln supplementation of conventional TPN acutely raises intramuscular free glutamine concentration by a small but significant amount although a longer period of Ala-Gln provision would probably be necessary to raise glutamine concentrations further. Ala-Gln supplementation also appears to raise muscle

protein synthetic rate (by 88%), although absolute values only approach the range seen in healthy *fasted* man (see Chapter 3) and still remain below values observed (by historical comparison) when a physiological mixture of amino acids similar to that used in the present studies was administered to healthy subjects (Bennet *et al.*, 1989). In this study no correlation could be demonstrated between intramuscular glutamine concentration and muscle protein synthetic rate. However, it should be noted that the overall subject numbers used in this calculation of correlation in man were small in comparison to the large numbers used in a previous animal study demonstrating a significant positive correlation (Jepson *et al*, 1988).

The mechanism by which glutamine supplementation exerts its observed effect in raising muscle protein synthesis in post-operative patients is unknown. Nor has it been possible to determine whether the effect is a direct effect at cellular level or indirectly mediated. However, the possible hormonal agents which may play a role in any indirect effect glutamine may have on protein synthesis and which were monitored in the present studies were unchanged in their plasma concentrations between the two phases of the longitudinal study. Branched chain amino acids and leucine in particular, previously reported to have a regulatory role in muscle protein metabolism (Buse and Reid, 1975) also do not appear to play a significant part as concentrations were similar with or without Ala-Gln infusion. Glutamine appears to be conditionally essential for the maintenance of muscle protein synthesis in post-operative patients and the overall amelioration in whole-body nitrogen balance observed in previous studies of Ala-Gln provision (Stehle *et al.*, 1989; Hammarqvist *et al.*, 1989) to post-operative subjects may in part be explained by a stimulatory effect which

increased glutamine appears to have on muscle protein synthetic rate. However tracer incorporation methodology as used in the present study can only provide partial information and it would be essential to carry out further studies of arteriovenous tracer exchange for a more complete understanding of glutamine's net effect on post-operative muscle protein balance.

CHAPTER 6

THE EFFECT OF L-ALANYL-L-GLUTAMINE SUPPLEMENTATION OF CONVENTIONAL TPN ON SKELETAL MUSCLE PROTEIN SYNTHESIS IN POST-SURGICAL PATIENTS: STUDIES OF [^{15}N]PHENYLALANINE ARTERIOVENOUS EXCHANGE

CONTENT

TABLES

FIGURES

6.1 SUMMARY

The acute effects of L-alanyl-L-glutamine (Ala-Gln) dipeptide supplementation of conventional TPN on skeletal muscle protein synthesis and breakdown have been investigated in post-operative patients. Sixteen patients received a constant infusion of non-glutamine containing TPN from the first post-operative day up to and including the third post-operative day when muscle protein synthesis and breakdown were measured over an eight hour period using a primed constant infusion of [^{15}N]phenylalanine with determination of forearm arteriovenous plasma phenylalanine balance and ^{15}N enrichment. The effects of TPN alone were studied in eight of sixteen patients ('cross-sectional' study of Ala-Gln supplementation); the remaining eight patients were studied over two 4-hour periods ('longitudinal' study of Ala-Gln supplementation). In the first period, TPN was supplemented with a primed constant infusion of L-alanine plus glycine (Ala+Gly) as a control mixture isonitrogenous with the primed constant infusion of Ala-Gln given in the second period.

The mean plasma arterial glutamine concentration between 90-480 min in the cross-sectional study was 340±11 (311, 370) μmol/l (mean±SEM, (95% confidence intervals)). In the longitudinal study the mean glutamine concentration during infusion with TPN + Ala+Gly was 314±16 (274, 355) μmol/l, rising by 29% to 404.4±30 (316, 471) μmol/l ($p<0.05$) with the infusion of TPN + Ala-Gln. Forearm mixed protein breakdown in the cross-sectional study patients was 32.8±5 (21, 44) nmol/100g/min; the values remained similar during Ala+Gly and Ala-Gln supplementation at 37.6±11 (9, 67), 36.7±9 (14, 59) nmol/100g/min respectively. 3-methylhistidine efflux in the cross-sectional group was -0.24±0.5 (-1.5, 1.0)

nmol/100g/min. Efflux of 3-methylhistidine was -0.6±0.2 (-1.2, 0.1) nmol/100g/min during Ala+Gly supplementation, increasing with Ala-Gln infusion to -0.9±0.3 (-2, -0.1) nmol/100g/min ($p<0.02$). Forearm protein synthesis was 19.1±3 (12, 26) nmol/100g/min in the cross-sectional study, similar to the rate of 17.8±2 (12, 23) nmol/100g/min during Ala+Gly infusion, increasing to 23.4±2 (18, 29) nmol/100g/min ($p<0.03$, 1-tailed) during the Ala-Gln period. The net negative protein balance decreased by 32.5% from the Ala+Gly to the Ala-Gln period (-20±5 (-33, -7) *versus* -13.5±5 (-27, 0.4) nmol/100g/min), although this change did not reach significance at the 5% level. In the cross-sectional study whole body phenylalanine flux and protein breakdown were 62±4 (52, 72) and 45.8±4 (37, 54) μmol/kg/h respectively. Whole-body phenylalanine flux was 63.5±2 (58, 69) μmol/kg/h, TPN + Ala+Gly, decreasing slightly to 60.5±4 (52, 69) μmol/kg/h, TPN + Ala-Gln. Whole-body protein breakdown decreased slightly but not significantly from the period of Ala+Gly supplementation (47.7±2 (43, 52) μmol/kg/h), to the period of Ala-Gln supplementation (44.7±3 (37, 52) μmol/kg/h). These results suggest that Ala-Gln supplementation of TPN in post-operative patients increased availability of glutamine and acutely stimulated muscle mixed protein synthesis, assessed by [^{15}N]phenylalanine arteriovenous exchange, without altering the rate of mixed protein breakdown. Increased 3-methylhistidine efflux with Ala-Gln supplementation appeared to indicate that the rate of *myofibrillar* protein breakdown was increased by glutamine dipeptide supplementation in these patients following major elective surgery, in whom both muscle protein synthesis and protein breakdown are usually reduced from pre-operative values. However the likelihood of trans-stimulation of the 3-methylhistidine transporter by the large glutamine provision, leading to increased efflux of the amino acid, could make this

interpretation unreliable.

6.2 Introduction

Studies of protein balance in man, using techniques involving measurement of arteriovenous exchange of amino acids across limbs, indicate that following injury (including surgical injury), trauma and sepsis, there is a net efflux from skeletal muscle of certain amino acids, chiefly the gluconeogenic amino acids glutamine and alanine (Aulick and Wilmore, 1979; Clowes *et al.*, 1980). There is also efflux of the amino acid 3-methylhistidine from skeletal muscle myofibrillar protein, and this has been used as the basis of a quantitative assessment of myofibrillar protein breakdown (Ballard and Tomas, 1983). Conventional TPN does not appear to reverse the typically negative post-operative nitrogen balance and is unable to increase post-operative muscle protein synthesis as determined by the 'flooding dose' technique (Essén, 1991). Also, it does not appear to improve amino acid and protein balance as measured by limb arteriovenous exchange (Möller-Loswick, *et al.*, 1991). Various methods of nutritional and hormonal supplementation of conventional TPN have been advocated and investigated to establish a possible role for such agents in augmenting the efficacy of TPN in improving peri-operative whole-body nitrogen and protein balance. Human recombinant growth hormone has been shown to improve post-operative nitrogen balance and to stimulate post-operative muscle protein synthesis as determined by [^{13}C]leucine tracer incorporation studies (Ponting *et al.*, 1988). Branched chain amino acid-enriched TPN however did not improve post-operative muscle protein

synthesis when assessed *indirectly* by the semi-quantitative method of muscle polyribosome concentration determination (Hammarqvist *et al.*, 1988). Glutamine supplementation of conventional TPN did improve post-operative nitrogen balance and reduced the extent of the post-operative fall in intramuscular free glutamine concentration and the associated fall in muscle protein synthetic rate determined by intramuscular polyribosome concentration (Hammarqvist *et al.*, 1989). However, to our knowledge, no studies have been reported to date which have sought to establish by *direct* methodology the effect of glutamine provision on muscle protein balance, i.e. synthesis *and* breakdown, in post-operative patients. Although tracer incorporation studies using [^{13}C]leucine and tissue biopsy can be used to measure protein synthesis, they do not provide information on protein *breakdown*. Information on both protein synthesis and breakdown is required to assess the net effect of glutamine, administered either in free form or as the dipeptide Ala-Gln, on muscle protein balance and to formulate an explanation for the mechanisms by which the previously reported net improvement in nitrogen balance occurs.

The amino acid L-phenylalanine is neither synthesised nor metabolised by skeletal muscle. Stable isotope labelled [^{15}N]phenylalanine is therefore an ideal tracer for use in the investigation of muscle protein synthesis and breakdown. 3-methylhistidine (i.e. N^{τ}-methylhistidine) is an amino acid produced by post-translational methylation at the N^{τ} position of histidine-73 of the actin chain (Vanderkerckhove and Webster, 1984). When released by the breakdown of myofibrillar proteins actin and myosin, 3-methylhistidine is not reincorporated into protein by protein synthesis and does not undergo intermediary metabolism (Young *et al.*, 1971). Actin and myosin together constitute 50% of muscle protein and release of 3-methylhistidine therefore provides a specific index of breakdown of

these myofibrillar proteins. The arteriovenous balance of 3-methylhistidine across the forearm has been measured in this study to determine the effect of Ala-Gln provision on post-operative myofibrillar protein breakdown.

6.3 Methods

6.3.1 Place of Study

All studies were performed in the intensive care unit of Ninewells Hospital and Medical School, Dundee.

6.3.2 Patients

The present studies were performed concurrently with the studies of [1-^{13}C]leucine tracer incorporation described in Chapter 5 and the same patients were investigated for each study i.e. 8 patients for the 4 h - 4 h longitudinal study of Ala-Gln supplementation of TPN and 8 patients for the 8 h (TPN alone) cross-sectional study. All patients had undergone potentially curative upper gastrointestinal surgery for carcinoma of the oesophagus and were studied on their third post-operative day. Their ages, morphological data and operative details are as detailed in Tables 5.1a and 5.1b, Chapter 5.

Ethical approval for these studies was obtained from the Tayside Committee on Ethics in Medical Research. Written informed consent was obtained from each

patient after a 2 hour interview and provision of a written information sheet.

6.3.3 Study Protocol

The overall design used for the studies described here is as described in Protocol 2 and 3, Chapter 2.

6.3.4 TPN Solution

From the first post-operative day till the third post-operative day when the metabolic studies described here were performed, patients received TPN in the form of a 2.5 l bag of solution per 24 h. The solution comprised a mixture of two commercially available mixed amino acid solutions: 500 ml of Synthamin 14 (14 g amino-N per litre) and 500 ml of Synthamin 9 (9 g amino-N per litre) (Baxter Healthcare, Thetford, England), providing 11.5 g amino-N, 1500 ml glucose 30% (Baxter Healthcare, Thetford, England) providing 7532 kJ, and replacement electrolytes (70 mmol Na^+, 80 mmol K^+).

6.3.5 Tracer Solution

A pre-sterilised solution of [^{15}N]phenylalanine (99 atom%, Tracer

Technologies, U.S.A.), in 0.9% saline (Baxter Healthcare, Thetford, England) was infused as a primed constant infusion at a rate of 0.5 mg (amino acid)/kg body weight/hour respectively, after a 0.5 mg/kg priming dose.

6.3.6 Study Solutions

Pre-sterilised solutions (prepared in the Department of Pharmacy, Ninewells Hospital and Medical School, Dundee) of the free amino acids L-alanine and glycine (Kabi-Vitrum, Sweden), in 250 ml 0.9% saline (Baxter Healthcare, Thetford, England) were infused at 4.8 mg/kg/h and 8.0 mg/kg/h respectively (after priming bolus doses of 4.8 mg/kg and 8.0 mg/kg respectively in a volume of 20 ml 0.9% saline), providing a total of 2.25 mg amino-N/kg/h, for 4 h during the first half of the 8 h tracer infusion study. A sterile solution of the dipeptide L-alanyl-L-glutamine (Ala-Gln, Kabi-Pfrimmer, Germany) in 250 ml 0.9% saline (Baxter Healthcare, Thetford, England) was infused at 11.7 mg/kg/h (after a priming bolus of 11.7 mg/kg in 20 ml 0.9% saline), providing 2.25 mg amino-N/kg/h, for 4 h during the second half of the 8 h tracer infusion study.

6.3.7 Siting of Cannulas

Deep venous blood was obtained from the "study" forearm from a 18 gauge cannula (Venflon, Viggo, Sweden) inserted retrogradely into the median cephalic or basilic ante-cubital vein. A paediatric sphygmomanometer cuff was applied to

the wrist and inflated to above systolic pressure for one minute prior to blood sampling in order to minimise the variable effects of the cutaneous circulation, minimising the admixture of deep venous blood (draining predominantly the forearm muscle bed) with cutaneous blood from the hand. Before commencing the main study, confirmatory evidence that this venous cannula was indeed draining the deep tissues i.e. the muscle bed of the forearm, was obtained by measurement of blood gases of blood sampled from the cannula. A O_2 saturation value of 50% or below was assumed to indicate the presence of blood draining the deep tissues (Jackson *et al.*, 1987). Arterial blood was obtained from an intra-arterial cannula (Viggo, Sweden) sited in the radial artery (at the wrist) for routine post-operative monitoring of mean arterial blood pressure. A triple lumen catheter was inserted in the internal jugular vein at the time of surgery by the senior anaesthetist in charge. This cannula was used initially for monitoring of central venous pressure and for administration of inotropic drugs if required as well as for TPN infusion. On the study day one lumen was used for the infusion of the study (amino acid and dipeptide) solutions.

6.3.8 Blood Flow Measurement

Blood flow was measured in the study forearm using venous occlusion strain gauge plethysmography using a commercially available machine (Medimatic, Denmark) regularly maintained and calibrated by the Vascular Laboratory, Ninewells Hospital and Medical School, Dundee and kindly lent for the present metabolic studies. Appropriate precautions were taken according to guidelines

outlined by Jackson and colleagues (Jackson *et al.*, 1987) with, in particular, a paediatric wrist cuff being applied, as described above, prior to blood flow measurement in order to stabilise the measured blood flow by minimising the variable effects of the cutaneous circulation on flow.

6.3.9 Calculations

The ^{15}N enrichment of arterial and venous plasma phenylalanine during the primed continuous infusion of [^{15}N]phenylalanine and plasma concentrations of phenylalanine were used to calculate phenylalanine exchange by limb tissue from the degree of dilution of the tracer in the venous outflow in comparison with the arterial inflow. The equations used are detailed in Chapter 2.

6.4 RESULTS

6.4.1 Arterial Plasma Amino Acid Concentrations

The concentrations of free amino acids in arterial plasma, and the changes in their concentrations from basal values to values attained during TPN infusion alone or TPN supplemented by either an L-alanine *plus* glycine mixture or by L-alanyl-L-glutamine are detailed and discussed in part in the previous chapter (Chapter 5, section 5.4.1 and Tables 5.2, 5.3). In particular, in the longitudinal study, the mean plasma arterial glutamine concentration (Chapter 5, Figure 5.1

and Table 5.2) was 329.5±16 (281, 364) μmol/l (mean±SEM, 95% confidence intervals) from the period -30 min to 0 min, falling slightly to 313±16 (274, 355) μmol/l during infusion with Ala+Gly, although this change was not statistically significant; with the infusion of Ala-Gln, the glutamine concentration rose by 23% to 404.4±30 (316, 471) μmol/l ($p<0.05$). The plasma concentration of phenylalanine was stable during each phase of the longitudinal study (Figure 6.1). The mean phenylalanine concentrations in the longitudinal study for the basal, Ala+Gly and Ala-Gln periods were stable at 98±7 (79, 116) μmol/l, 93±5 (80, 106) μmol/l and 96±4 (82, 110) μmol/l. The corresponding basal and 90-480 min values for the cross-sectional study group, comparing with the basal and Ala+Gly periods of the longitudinal study, were higher at 118±9 (98, 139) μmol/l and 115±8 (97, 134) μmol/l respectively, although the differences were not significant. The mean basal tyrosine concentration in the longitudinal study was 54.3±7 (34, 71) μmol/l ($p<0.02$), falling by 10% during Ala+Gly infusion to 48.6±6 (32, 64) μmol/l and by a further 5% during the Ala-Gln period to 46.3±5 (30, 60) μmol/l). The basal and 90-480 min tyrosine concentrations in the cross-sectional study group, compared with the basal and Ala+Gly periods of the longitudinal study, were similar at 67±4 (57, 76) μmol/l and 65±4 (55, 75) μmol/l respectively. The mean basal 3-methylhistidine concentration in the longitudinal study was 3.71±0.4 (2.8, 5.1) μmol/l, 3.57±0.3 (3, 4.9) μmol/l during Ala+Gly infusion and 3.0±0.4 (1.7, 4.1) μmol/l during the Ala-Gln period. There were no significant differences in the corresponding basal and 90-480 min values for the cross-sectional study group, which were similar at 3.88±0.6 (2.4, 5.4) μmol/l and 3.75±0.6 (2.2, 5.4) μmol/l respectively.

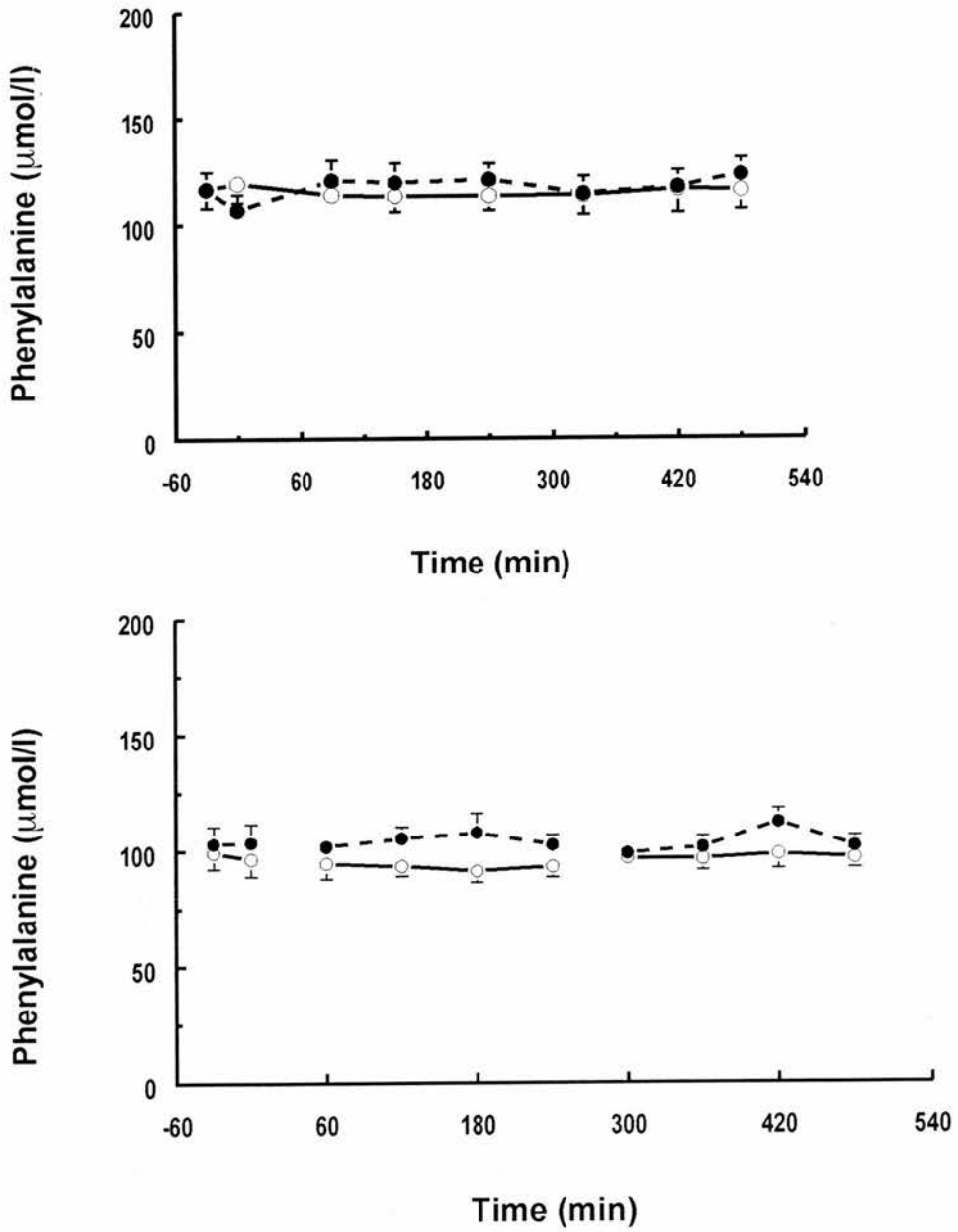

Figure 6.1 Plasma arterial (O) and venous (●) concentration of free phenylalanine in patients receiving TPN alone (90-480 min, top graph) and in patients receiving TPN supplemented by either Ala+Gly (60-240 min) or Ala-Gln (300-480 min, bottom graph).

6.4.2 Forearm Amino Acid Balance

In the longitudinal study the net effluxes from the forearm of phenylalanine and tyrosine (Table 6.1a) during the Ala+Gly period were -20±5 (-33, -7) and -16.7±4 (-29.5, -4) nmol/100g/min respectively. The phenylalanine efflux decreased to -13.5±5 (-27, -0.4) nmol/100g/min during the Ala-Gln period. The tyrosine efflux did not significantly change at -20.1±4 (-33.5, -6.5) nmol/100g/min. In the cross-sectional study, net effluxes of phenylalanine and tyrosine between 90-480 min (Table 6.1a) were -13.6±4 (-23, -4) and -18.6±7 (-36, -2) nmol/100g/min respectively: these values, also, were not significantly different to the values observed during either of the Ala-Gln or the Ala+Gly infusion periods in the longitudinal study. The efflux of 3-methylhistidine from the forearm increased with Ala-Gln infusion, from -0.6±0.2 (-1.2, 0.1) nmol/100g/min (with Ala+Gly) to -0.9±0.3 (-2, -0.1) nmol/100g/min ($p<0.02$) which would *appear* to suggest that the rate of myofibrillar protein breakdown was increased by glutamine dipeptide supplementation. In comparison to 3-methylhistidine efflux during the Ala+Gly period the corresponding value at plateau in the cross-sectional study (Table 6.1a) was not significantly different at -0.24±0.5 (-1.5, 1.0) nmol/100g/min.

Total amino acid efflux increased slightly from -618±111 (-838, -398) nmol/100g/min with TPN + Ala+Gly to -643±115 (-870, -416) nmol/100g/min with TPN + Ala-Gln. The total efflux of essential amino acids fell from -206.1±43 (-292, -398) nmol/100g/min with TPN + Ala+Gly to -180.6±31 (-243, -118) nmol/100g/min with TPN + Ala-Gln. The total efflux of non-essential amino acids increased from -411.5±62 (-310, -62) nmol/100g/min with TPN + Ala+Gly to -462±110 (-682, -243) nmol/100g/min with TPN + Ala-Gln. However, excluding the

supplemental amino acids Ala, Gln and Gly, the total efflux of the remaining non-essential amino acids fell from -70.7±30 (-131, -11) nmol/100g/min with TPN + Ala+Gly to -40.3±25 (-90, -9) nmol/100g/min with TPN + Ala-Gln.

6.4.3 Plasma Hormones and Substrates

The concentrations of plasma C-peptide, insulin, glucagon, IGF-I and of blood D-(-)-3-hydroxybutyrate were stable within each study period in the longitudinal study (Chapter 5, Table 5.6).

6.4.4 Plasma [^{15}N] Enrichment of Phenylalanine

Plateau was attained for the enrichment of plasma phenylalanine during the final periods of each study (Figure 6.2) and in particular, during each phase of the longitudinal study. In the latter study, arterial plasma phenylalanine enrichment (APE) was 4.2±0.2 (3.7, 4.7) during the Ala+Gly period, rising to 4.5±0.3 (3.8, 5.1), $p<0.05$, during Ala-Gln infusion.

Table 6.1a Arterio-venous balance of plasma amino acids. CI = 95% confidence intervals.

Amino acid	TPN alone (90-480 min) nmol/100g/min	CI	TPN + Ala+Gly (60-240 min) nmol/100g/min	CI	TPN + Ala-Gln (300-480 min) nmol/100g/min	CI
ALA	-203.7 ± 42	(-311, -97)	-161.3 ± 35	(-270, -53)	-202.6 ± 41	(-341, -64)
ARG	-21.3 ± 5	(-35, -8)	-35.4 ± 13	(-75, 4)	-33.9 ± 6	(-55, -13)
ASP	-5.9 ± 4	(-17, 5)	2.9 ± 2	(-2, 8)	3.0 ± 2	(-5, 11)
ASN	-31.6 ± 9	(-55, -9)	-18.9 ± 7	(-39, 1)	-21.8 ± 5	(-39, -5)
GLU	48.6 ± 11	(21, 77)	39.4 ± 5	(24, 55)	62.0 ± 12	(24, 100)
GLN	-191.7 ± 53	(-329, -54)	-176.9 ± 45	(-317, -37)	-256.4 ± 37	(-392, -121)
GLY	-47.7 ± 17	(-92, -3.6)	-61.7 ± 37	(-231, 67)	-93.4 ± 14	(-138, -49)
HIS	-17.7 ± 6	(-57, -72)	-22.5 ± 7	(-42, -31)	-22.9 ± 4	(-37, -9)
3-MH	-0.2 ± 0.5	(-1.5, 1)	-0.6 ± 0.2	(-1.2, 0.1)	-0.9 ± 0.3	(-2, -0.1)
ISO	-10.8 ± 9	(-33, 11)	-11.4 ± 5	(-27, 3)	-10.2 ± 2	(-18, -2)
LEU	-14.3 ± 17	(-58, -29)	-22.8 ± 11	(-69, 24)	-12.9 ± 1	(-16, -9)
LYS	-70 ± 18	(-117, -23)	-74.8 ± 21	(-139, -105)	-65.5 ± 9	(-97, -34)
MET	-22.4 ± 13	(-156, 35.1)	-17.0 ± 5	(-31, -3)	-13.1 ± 3	(-21, -5)
PHE	-13.6 ± 4	(-23, -4)	-20 ± 5	(-33, -7)	-13.5 ± 5	(-27, 0.4)
SER	-14.6 ± 11	(-42, 13)	-25.1 ± 12	(-62, 12)	-28.1 ± 3	(-40, -17)
TAU	-3.2 ± 2	(-10, 3)	-5.6 ± 2.5	(-13, 2)	-0.3 ± 3	(-9, 8)
THR	-35.2 ± 17	(-79, 9)	-32.6 ± 10	(-63, -2)	-33.4 ± 5	(-52, -15)
TYR	-18.6± 7	(-36, -2)	-16.7 ± 4	(-30, -4)	-20.1 ± 4	(-34, -7)
TRY	-16.1 ± 7	(-33, 1)	-4.1 ± 2	(-11, 3)	-5.8 ± 1	(-9, -3)
VAL	-38.1 ± 5	(-52, -25)	-22.3 ± 13	(-63, 18.5)	-16.8 ± 16	(-69, 35)
BCAA	-63.7 ± 34	(-152, 25)	-69.9 ± 33	(-171, 31)	-55.2 ± 23	(-127, 17)

Table 6.1b Total arterio-venous balance of plasma amino acids (longitudinal study). CI = 95% confidence intervals.

	TPN + Ala+Gly (60-240 min)		TPN + Ala-Gln (300-480 min)	
	nmol/100g/min	CI	nmol/100g/min	CI
Total amino acids	-618.0 ± 110	(-838, -398)	-643.0 ± 115	(-870, -416)
Total essential amino acids	-206.1 ± 43	(-292, -120)	-180.6 ± 31	(-243, -118)
Total non-essential amino acids	-411.5 ± 62	(-310, -62)	-462 ± 110	(-682, -243)
Total non-essential amino acids, - excluding ALA, GLN, GLY	-70.7 ± 30	(-131, -11)	-40.3 ± 25	(-90, -9)

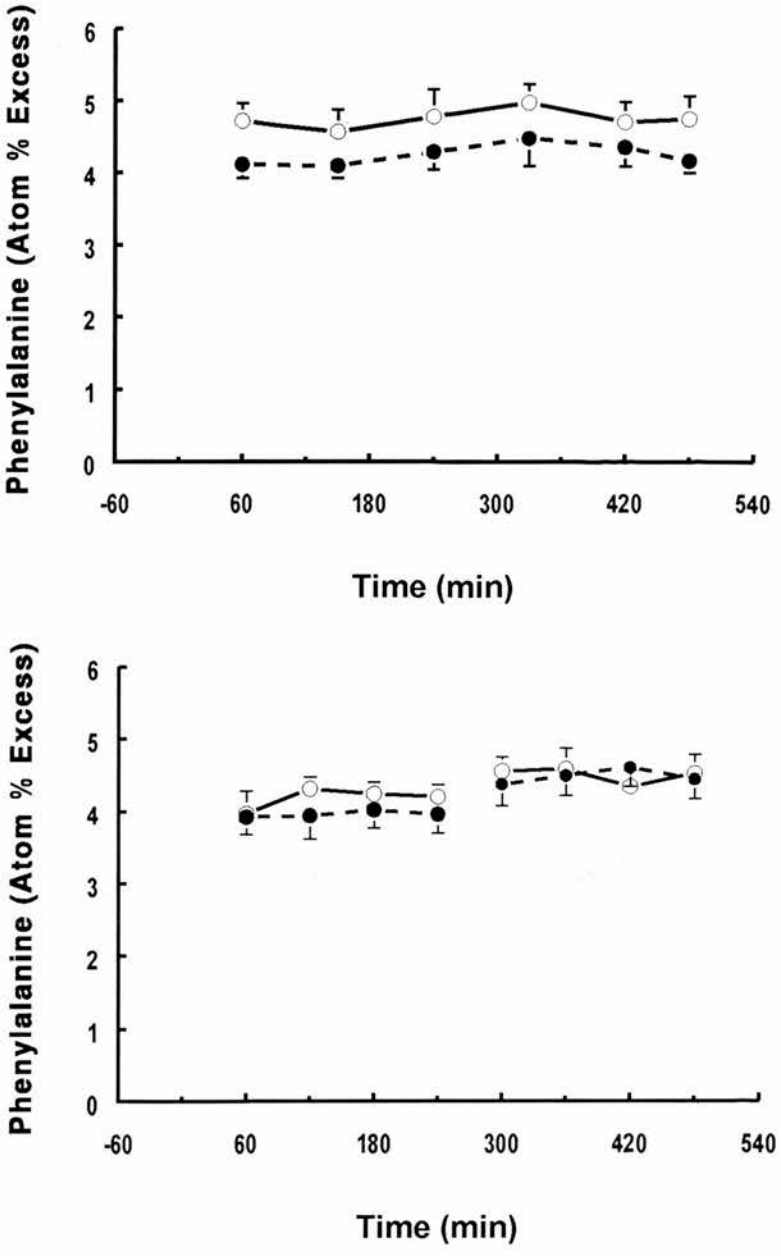

Figure 6.2 Plasma arterial (○) and venous (●) enrichment of free phenylalanine in patients receiving TPN alone (90-480 min, top graph) and in patients receiving TPN supplemented by either Ala+Gly (0-240 min) or Ala-Gln (240-480 min, bottom graph).

6.4.5 Forearm Protein Balance by [^{15}N]phenylalanine Arteriovenous Exchange

In the longitudinal study forearm mixed protein breakdown, assessed by[^{15}N]phenylalanine, was similar during the Ala+Gly and Ala-Gln periods at 37.8±7 (20, 55) nmol/100g/min *versus* 36.9±7 (22, 51) nmol/100g/min respectively (Figure 6.3). The protein breakdown rate in the cross-sectional study was also similar at 32.8±5 (21, 44) nmol/100g/min. Forearm protein synthesis increased from 17.8±2 (12, 23) nmol/100g/min during the Ala+Gly period of the longitudinal study to 23.4±2 (18, 29) nmol/100g/min ($p<0.03$, 1-tailed) during the Ala-Gln period (Figure 6.4). Forearm protein synthesis in the cross-sectional study was similar to the value during the Ala+Gly period of the longitudinal study, at 19.1±3 (12, 26) nmol/100g/min. The balance between protein synthesis and protein breakdown decreased by 32.5% from the Ala+Gly to the Ala-Gln period, although this change did not reach statistical significance.

6.4.6 Whole-body Phenylalanine Kinetics

In the longitudinal study, whole-body phenylalanine flux (Figure 6.5) fell from 63.5±2 (58, 69) μmol/kg/h during the Ala+Gly period to 60.5±4 (52, 69) μmol/kg/h during the Ala-Gln period, although the change was not statistically significant. During the same periods whole-body protein breakdown decreased slightly but not significantly from 47.7±2 (43, 52) μmol/kg/h to 44.7±3 (37, 52) μmol/kg/h respectively. In the cross-sectional study (Figure 6.5), whole body

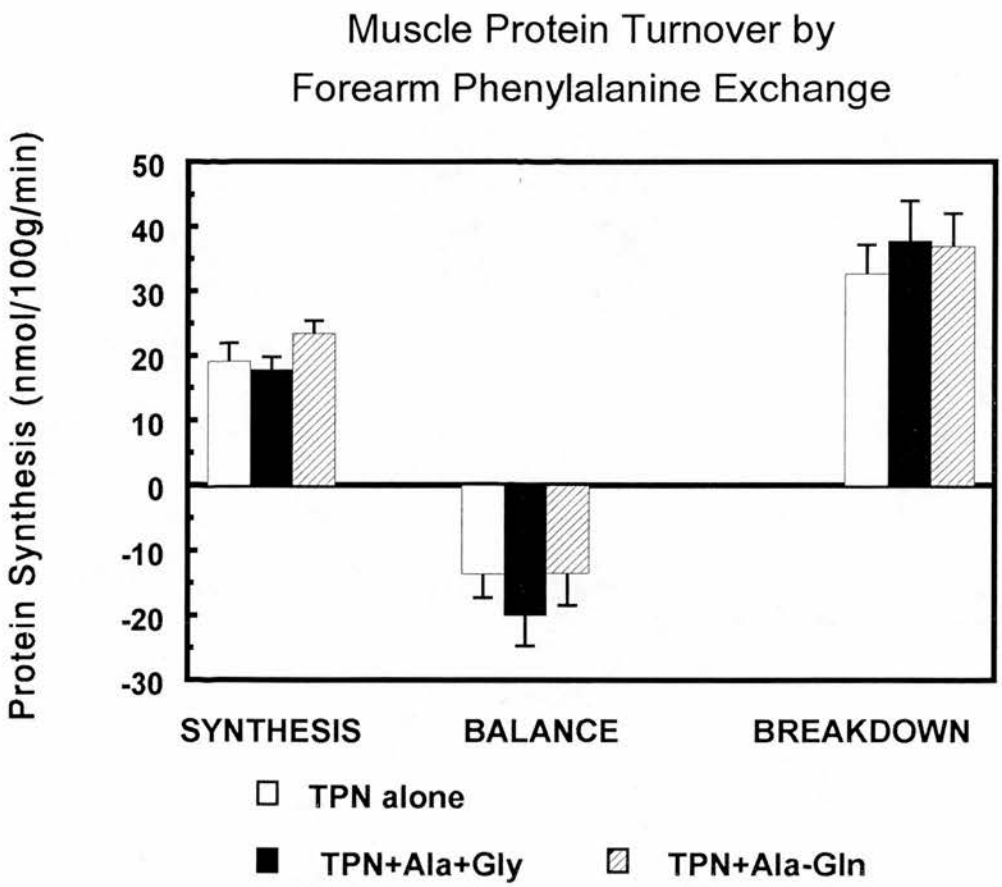

Figure 6.3 Components of forearm phenylalanine metabolism in the patient group receiving TPN alone (unfilled bar) and the group receiving TPN supplemented by Ala+Gly (filled bar) and Ala-Gln (hatched bar).

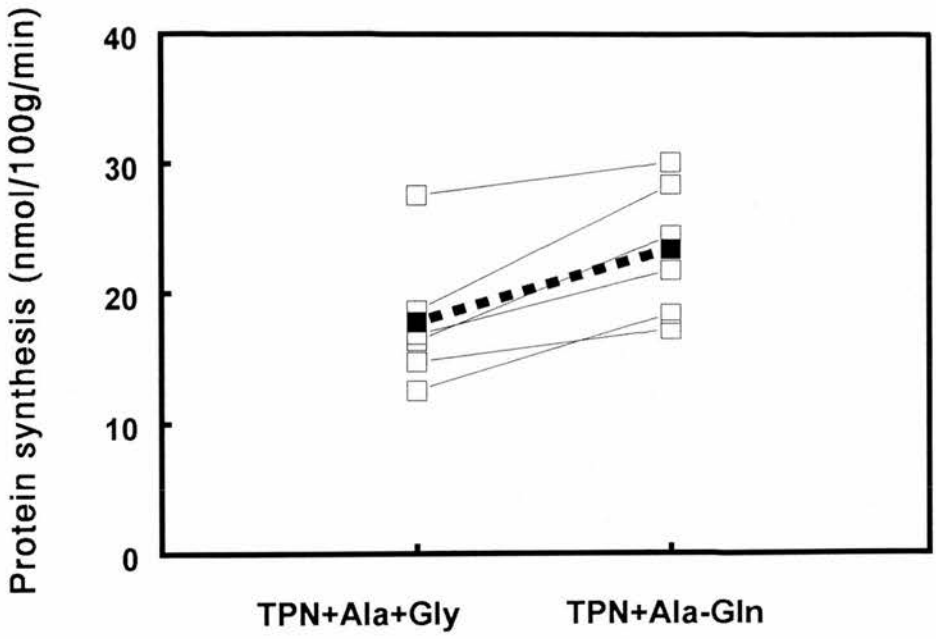

Figure 6.4 Skeletal muscle protein synthesis in the patient group receiving TPN supplemented either by Ala+Gly (on the left) or Ala-Gln (hollow squares: individual values; solid squares: mean values).

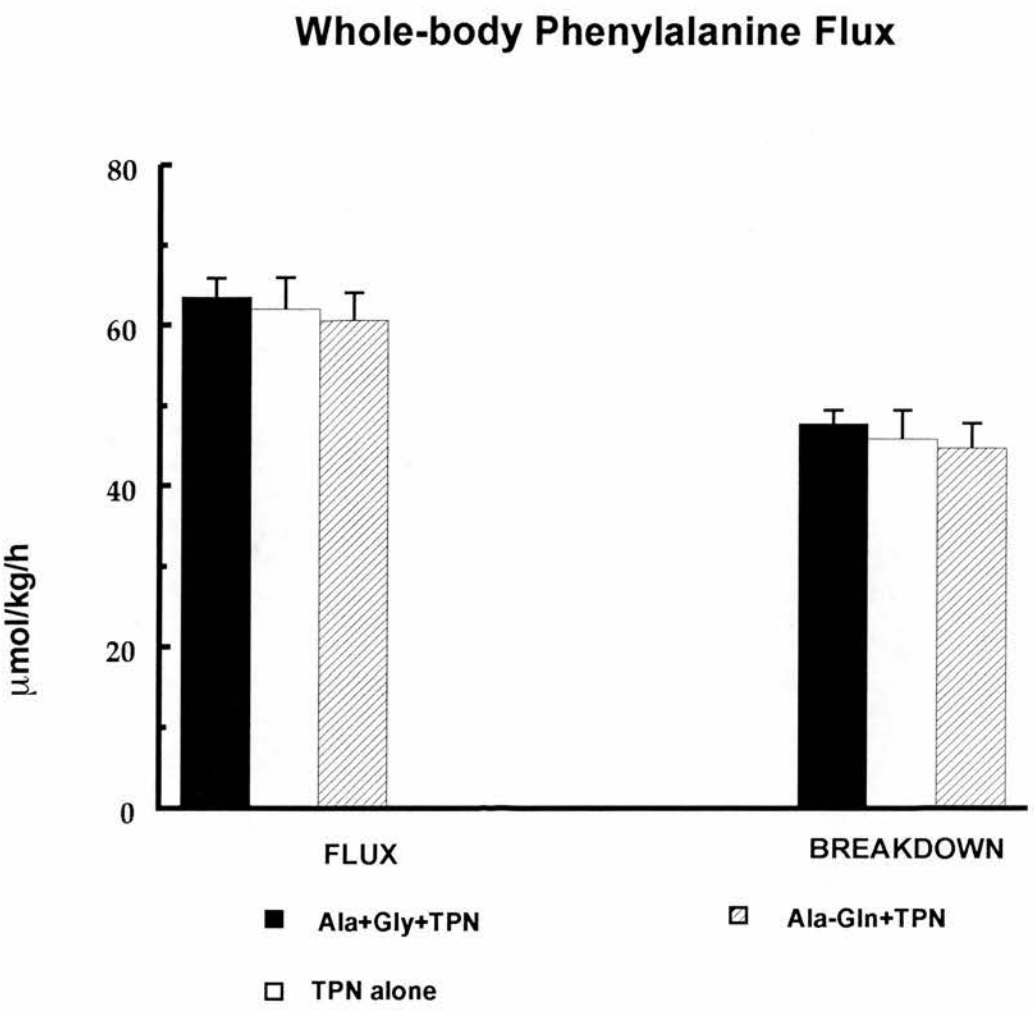

Figure 6.5 Whole-body phenylalanine flux and breakdown for the patient group receiving TPN alone (unfilled bar) and the group receiving TPN supplemented by Ala+Gly (filled bar) and Ala-Gln (hatched bar).

phenylalanine flux and protein breakdown during 90-480 min were similar at 62±4 (52, 72) μmol/kg/h and 45.8±4 (37, 54) μmol/kg/h respectively.

6.5 DISCUSSION

The potential pitfalls of a 4 h - 4 h periods longitudinal study design have been discussed in Chapter 5 with particular reference to the [1-^{13}C]leucine incorporation method for studying protein synthesis by the primed constant infusion technique. When studying muscle protein turnover by the [^{15}N]phenylalanine arteriovenous exchange method it is equally important to attain steady states of tracer enrichment during each study period, as the model used for calculating protein synthesis and breakdown presupposes such steady states. Steady states were indeed achieved for [^{15}N] enrichment of phenylalanine and also of phenylalanine concentration during both 4 hour periods of the longitudinal study and also during the 8 hour cross-sectional study. The principal advantage of the longitudinal study protocol, i.e. using individual patients as their own control in order to minimise the effects of variables on protein synthesis such as the heterogenous pathophysiological response to surgery, anaesthesia and TPN, is particularly pertinent to the present study where the methodology itself is subject to the influence of additional important variables e.g. the measured blood flow in the forearm muscle bed and the fluctuations in the venous drainage from this muscle bed (Butler and Home, 1987). Thus the coefficients of variation of the measures of protein turnover were larger with the use of the [^{15}N]phenylalanine arteriovenous exchange method than the variation observed for muscle protein

synthesis calculated from tissue enrichments in samples obtained by biopsy. This was probably due to errors compounding in the calculations based on small arteriovenous differences and to the inter-individual variation in blood flow measurements, a major component in the calculation of protein turnover by the limb exchange method.

Although the clinical investigative methodology used in the present studies is well established and has been previously used and adapted at our centre by Bennet and colleagues (Bennet *et al.*, 1990) to investigate the effects of insulin and mixed amino acid provision on skeletal muscle protein synthesis and breakdown, Bennet's studies were performed on healthy volunteers and fully ambulant diabetic patients. The present studies were carried out on non-ambulant post-operative patients in the intensive care unit. These patients are at increased risk of deep venous thrombosis (DVT) (Barua, 1992) and it was therefore felt that femoral vein cannulation to obtain venous samples draining the lower limb muscles (Bennet *et al.*, 1990), would be wholly inappropriate, particularly for research purposes, as even relatively minor local endothelial injury at the site of cannulation could augment the risk of DVT. The forearm was therefore used for these studies as described by Cheng and colleagues (Cheng *et al.*, 1985). A potential disadvantage of using the forearm preparation however is that protein turnover values are determined for a much smaller muscle mass, possibly introducing greater error when extrapolating to calculate changes in protein turnover for the whole-body muscle mass.

The values of post-operative forearm arteriovenous balance of the amino acids alanine, glutamine, methionine and phenylalanine in the longitudinal and cross-sectional study groups are broadly in the same range as, but not identical to,

the values reported (with these amino acids alone being quoted) in a study investigating post-hysterectomy patients on TPN (Carli *et al.*, 1990). Although the forearm preparation has been used in both studies, the differences which exist in the amino acid balance results between the studies may be accounted for by differences in the patient groups (age, disease and nutritional state), the nature of surgical intervention and the subsequent metabolic response and the composition of the intravenous nutrition provided.

Muscle protein synthesis was significantly higher during the Ala-Gln period ($p<0.03$, paired t-test, 1 tailed) in comparison with the Ala+Gly supplementation period. Interestingly, the absolute values for muscle protein synthesis with TPN alone or Ala+Gly supplementation of TPN in post-operative patients were lower than that observed in the post-absorptive healthy subjects receiving the control solution of Ala+Gly, (19.1 ± 3 (12, 26) and 17.8 ± 2 (12, 23) *versus* 26.1 ± 2 (21, 32) nmol/100g/min) in the study described in chapter 4, and only approached parity with Ala-Gln supplementation (23.4 ± 2 (18, 29) *versus* 26.1 ± 2 (21, 32) nmol/100g/min), suggesting the presence, in post-operative patients, of a significant degree of residual resistance to the observed stimulatory effect of intravenous nutrition with TPN in healthy subjects (Bennet *et al.*, 1989). There was no significant difference in muscle protein breakdown between the first and second periods of the longitudinal study and this suggests that Ala-Gln supplementation of TPN does not alter mixed muscle protein breakdown in patients undergoing major surgery.

In relation to the arteriovenous balance of amino acids, one concern is that the observed increase in protein synthesis with Ala-Gln provision, without any significant increase in breakdown, does not appear to be reflected in a

corresponding decrease in total amino acid efflux. Although the reason for this apparent disparity is not self-evident, a number of factors can be considered which may be contributory. The co-efficient of variation in the measurement of arteriovenous differences was large and in this study this method is unlikely to have been sensitive for indirectly assessing quantitative changes in protein balance. The concentrations of phenylalanine, used in the calculations of balance and protein synthesis, were determined with mass spectrography methodology using true internal standards and in general are likely to have been more accurate than the concentrations of other amino acids determined by amino acid analyser with external standard. Also, the dipeptide Ala-Gln is metabolised in muscle to release into venous blood free Ala and Gln (Fürst, 1989), hence increasing 'production' and net efflux of these amino acids from muscle. Glycine provision was withdrawn in the TPN + Ala+Gln period and this could explain the increase in the net negative balance during this period. Excluding these three amino acids, the total efflux of non-essential amino acids fell by 43% with Ala-Gln supplementation and the total efflux of essential amino acids fell by 12%. Neither change, however, reached statistical significance.

The finding that the average muscle protein synthesis increased with Ala-Gln supplementation of TPN, by use of the [^{15}N]phenylalanine forearm arteriovenous exchange method, is in agreement with the results from a previous study in which protein synthetic rate was determined by [1-^{13}C]leucine tracer incorporation into percutaneous muscle biopsy samples (Chapter 5). The present findings show the effect of Ala-Gln supplementation of TPN to be in the same direction, although quantitatively to a lesser degree, as observed in data obtained by the tracer incorporation method. The quantitative difference could be accounted

for in part by differences between the two methodologies used, involving the use of two different stable isotope tracers. Also, the limb arteriovenous amino acid exchange method used in the present study may underestimate the actual intracellular values of protein synthesis and breakdown, as a fraction of the intracellular amino acids resulting from protein breakdown is directly reincorporated into protein without entering the plasma amino acid pool (Biolo *et al.*, 1995).

The processes which regulate muscle protein breakdown in patients undergoing elective surgery do not appear to be acutely influenced by provision of glutamine in the form of Ala-Gln. There is evidence that in certain conditions including elective surgery which involve acute muscle wasting, both the mechanisms of muscle protein synthesis and breakdown are depressed (Rennie, 1985), with synthesis being depressed to a greater extent than breakdown, resulting in net negative protein balance. In these circumstances, intervention such as glutamine provision may have a net anabolic effect on protein balance by a possible combination of stimulation, inhibition or sparing of the processes of synthesis and breakdown, with the net effect favouring synthesis over breakdown. The evidence from the present study would appear to suggest that glutamine provision does stimulate protein synthesis in post-operative patients. Also, in contrast to the situation reported in perfused murine skeletal muscle (McLelland *et al.*, 1988), where glutamine provision inhibited protein breakdown, it does not appear to alter mixed protein breakdown in post-operative man. The efflux of 3-methylhistidine increased with Ala-Gln supplementation. This observation appears to suggest that myofibrillar protein breakdown increased in these patients with glutamine provision. However the possibility exists that significant trans-

stimulation of the 3-methylhistidine transporter (Hundal *et al.*, 1987) occurred with the large amount of administered glutamine, thereby increasing 3-methylhistidine efflux, making interpretation of myofibrillar protein breakdown unreliable, as efflux of this amino acid in these circumstances would not necessarily reflect the rate of myofibrillar protein breakdown.

Whole-body phenylalanine flux was not significantly different in each of the study periods in the longitudinal study group in comparison with values observed in the cross-sectional study group. Qualitatively, however, there was a trend towards a reduction in whole-body flux with the dipeptide supplementation, as previously observed using [1-^{13}C]leucine tracer (see Chapter 5). Information on whole-body protein synthesis was unavailable as tyrosine flux was not measured in this study (see Chapter 2 for discussion).

The net skeletal muscle protein balance remained negative and this result is in concordance with nitrogen balance studies of glutamine provision, either as Ala-Gln (Stehle *et al.*, 1989) or as free glutamine (Hammarqvist *et al.*, 1989) where it has been reported that although glutamine supplementation of TPN improved nitrogen balance, it did not reverse the generally observed negative nitrogen balance.

CHAPTER 7

THE EFFECT OF L-ALANYL-L-GLUTAMINE SUPPLEMENTATION OF CONVENTIONAL TPN ON ALBUMIN SYNTHESIS IN POST-OPERATIVE PATIENTS

CONTENTS

TABLES

FIGURES

7.1 SUMMARY

The acute effects of L-alanyl-L-glutamine (Ala-Gln) dipeptide supplementation of conventional TPN on albumin synthesis have been investigated in patients undergoing major curative surgery for oesophageal carcinoma. Eight patients received a constant infusion of non-glutamine containing TPN from the first post-operative day up to and including the third post-operative day when albumin synthesis was measured over an eight hour period using a primed constant infusion of [1-^{13}C]leucine with determination of plasma [^{13}C]-α-ketoisocaproate ([^{13}C]-αKIC) enrichment and [^{13}C]leucine incorporation into plasma albumin. The eight patients were studied over two 4-hour periods. In the first, TPN was supplemented with a primed constant infusion of L-alanine plus glycine (Ala+Gly) as a control mixture isonitrogenous with the primed constant infusion of Ala-Gln given in the second period. The mean plasma arterial glutamine concentration was 314±16 (274, 355) μmol/l (mean±SEM (95% confidence intervals)) during infusion with Ala+Gly, rising to 404±30 (316, 471) μmol/l ($p<0.05$) with the infusion of Ala-Gln. There was a rise in plasma α-ketoisocaproate enrichment from 4.75±0.3 (4.0, 5.5) APE during Ala+Gly infusion to 6.11±0.6 (4.7, 7.5) APE during Ala-Gln infusion, although this change did not reach statistical significance ($p=0.066$). The [^{13}C]leucine enrichment in plasma albumin rose from (0.039±0.001 (0.037, 0.042) to 0.088±0.001 (0.085, 0.092), APE) during the period 60-240 min of TPN + Ala+Gly infusion ($p<0.00001$) and from (0.179±0.002 (0.174, 0.185) to 0.246±0.005 (0.234, 0.258), APE) during the period 60-240 min of TPN + Ala-Gln infusion ($p<0.00001$). The calculated albumin synthesis rate was 0.041±0.05 (0.29, 0.53) %/h during Ala+Gly supplementation of TPN and was 37% higher at

0.056±0.10 (0.30, 0.82) %/h with Ala-Gln supplementation. Although this difference was just beyond the limit of significance on analysis by the Student's paired *t*-test (two-tailed), at the 5% level (p=0.058), there was nevertheless a definite trend toward higher values of albumin synthesis with the dipeptide supplementation in comparison to the isonitrogenous Ala+Gly mixture, indicating that short-term glutamine provision, in the form of Ala-Gln dipeptide supplementation of TPN appears to have an acute stimulatory effect on albumin synthesis in post-operative patients.

7.2 Introduction

Albumin is a plasma protein of molecular weight 69,000 which is synthesised in the liver. In healthy adult man, the plasma albumin concentration is 35-50 g/l and the total exchangeable albumin pool is 4.0-5.0 g/kg bodyweight. 38-45% of this albumin is intravascular and much of the rest is in the skin (Ganong, 1981). Six to ten per cent is degraded per day and the degraded albumin is replaced by hepatic synthesis of 200-400 mg/kg/d. Because of its relatively small size albumin has an important role in the maintaining of plasma oncotic pressure (James and Coward, 1981). Albumin has been reported to have scavenging properties in relation to free radicals (Holt *et al.*, 1984) and improves prognosis in the sheep model of the adult respiratory distress syndrome (Emerson *et al.*, 1988). It also appears to have anticoagulant properties, inhibiting platelet aggregation and enhancing the inhibition of factor Xa by antithrombin III (Jorgensen and Stoffersen, 1980 and 1979). Albumin is also an important carrier protein in

plasma, binding many substances, and is a useful marker of nutritional status (Mullen *et al.*, 1979).

It appears that albumin synthesis cannot be maintained for any length of time with the re-direction of amino acids derived from intracellular hepatic protein breakdown, and *in vitro* experiments, perfusion studies and measurements of albumin synthesis *in vitro* have all demonstrated the importance of extra-hepatic amino acids in determining the rate of albumin synthesis (Morgan and Peters, 1971; Rothschild *et al.*, 1977). Until relatively recently, direct measurement of the synthesis rate of albumin has only been carried out by a few investigators, e.g. Tavill and co-workers (1968), but there is clear evidence that the provision of amino acids by infusion will enhance synthesis (Skillman *et al.*, 1976). The earlier work on synthesis rate depended on inferring the rate from measurements of the catabolic rate measured under steady state conditions. Nevertheless, it is clear that albumin synthesis is much more sensitive to protein intake than can be inferred simply from the plasma albumin concentration (Hoffenberg *et al.*, 1966).

Recently, there has been increased interest in the measurement of rates of plasma albumin synthesis and metabolism, as albumin concentration is an useful indicator of nutritional and metabolic status, which is known to fall in critically ill and malnourished patients (Fleck *et al.*, 1985; Hunter *et al.*, 1985). However knowledge of the albumin concentration alone may be of limited value, as it is the resultant of several processes including synthesis and breakdown, changes in extracellular fluid volume, and losses to the extravascular space (Fleck *et al.*, 1985).

It is known that the rate of albumin synthesis can be regulated by dietary protein intake (Kaysen *et al.*, 1989), is normalised by infusion of branched-chain

amino acid-enriched total parenteral nutrition in patients with cancer cachexia (Hunter *et al.*, 1989), and is sensitive to changes in insulin in diabetic rats (Peavy *et al.*, 1978 and 1985). There is conflicting evidence available on the effect of insulin in type I diabetic patients, who are reported to show either a decrease (DeFeo *et al.*, 1991) or no change (Pacy *et al.*, 1990) in the rate of albumin synthesis after insulin withdrawal.

We became interested in the effect of Ala-Gln supplementation of TPN on albumin synthesis after having completed earlier studies on the effect of Ala-Gln supplementation of TPN on skeletal muscle protein turnover in post-surgical patients (Chapters 5 and 6). Also, the general methodology for investigating albumin synthesis had recently become established locally in our laboratory and successful studies had been performed investigating a possible stimulatory role of the flooding dose method in protein synthesis, in particular in acutely stimulating albumin synthesis in healthy post-absorptive subjects (Smith *et al.*, 1993). Because plasma samples remained from the earlier studies on the role of Ala-Gln supplementation in muscle protein turnover, we made some additional measurements on these samples in order to complete this retrospective study on the acute effect of short-term Ala-Gln supplementation of TPN on albumin synthesis.

7.3 Materials and Methods

7.3.1 Experimental Design

A detailed experimental protocol is given in the previous chapters (see Protocol 3, Chapter 2 and Chapter 5) and the present work only describes basic essentials and additional details.

7.3.2 Patient Details and Place of Study

Eight patients, (66-79 years, 44-85 kg, mean APACHE II score 9.13, (Chapter 5, Table 5.1b) who had undergone curative resection for oesophageal carcinoma, were studied postoperatively. All patients were studied on the third post-operative day, in the intensive care unit at Ninewells Hospital and Medical School, Dundee.

7.3.3 TPN and Infusion of Ala+Gly, Ala-Gln and Tracer Solutions

From the first postoperative day, patients were given intravenous nutrition, consisting of a 50:50 1 litre mixture of two commercially available mixed amino acid solutions, Synthamin 9 and Synthamin 14 (Baxter Healthcare, Norfolk, England) providing 11.5 g N, plus 450g glucose, 70 mmol Na^+ and K^+, all in 2.5 l water/24 h. The patients were studied on the third postoperative day between

0800 h and 1600 h when they received a primed constant infusion of [1-^{13}C]leucine. The study protocol was divided into two four hour periods: in the first the conventional non-glutamine containing TPN was supplemented with alanine (4.8 mg/kg/h) and glycine (8 mg/kg/h) to provide amounts isonitrogenous with the dipeptide (Ala-Gln, 11.8 mg/kg/h) which was given in the second four hour period.

7.3.4 Blood Sampling and Storage

Blood samples were taken prior to the infusion of tracer (at -30 to 0 min) and at frequent intervals throughout the study, according to protocol 3 described in Chapter 2. Blood samples were immediately stored on ice prior to subsequent cold centrifugation and separation of plasma. The plasma was stored at -80°C until the time of analysis.

7.3.5 Laboratory Analysis

The laboratory analytical techniques used were as described by Smith *et al.* (1992) and detailed in Chapter 2. Plasma was separated from the blood and used for the determination of concentration and enrichment of [^{13}C]leucine and α-ketoisocaproic acid by standard methods, using gas chromatography-mass spectrometry (Rocchiccioli *et al.*, 1981; Schwenk *et al.*, 1984).

Albumin was isolated from 1 ml plasma by alcoholic extraction of the trichloroacetic acid-precipitated protein (Ballmer *et al.*, 1990; Korner and Debro,

1956). The alcohol was evaporated under nitrogen at 70°C using a TurboVap LV evaporator (Zymark). Contamination of the albumin by free amino acids was minimized by redissolving the protein in 0.3 N NaOH at 37°C for 30 min, then reprecipitating with ice-cold 2M $HClO_4$. Measurement of the ^{13}C labelling of leucine in albumin was carried out by routine methods involving acid hydrolysis of the protein, purification of the acid hydrolysate by ion-exchange chromatography, preparative gas chromatography, and isotope ratio-mass spectrometry of carboxyl-CO_2 (Smith *et al.*, 1988).

7.3.6 Methods of Calculation

The rates of albumin synthesis were calculated by standard equations for the constant infusion method (see Chapter 2 for details). In the calculation of albumin synthetic rate it is essential to take into account the lag period between the synthesis and processing of albumin by the liver and its eventual secretion into plasma, i.e. the secretion time (t_s). The relationship between precursor and product is displaced by this secretion time, which can be calculated by plotting the regression line of the linear portion of the [^{13}C]leucine incorporation into albumin and extrapolating to the basal enrichment.

Statistical analysis for hypothesis testing was by Students paired *t*-test, with significance being assigned at the 5% level. Additional statistical analysis is presented, both in the table and in the text, in the form of 95% confidence intervals around the mean.

7.4 Results

7.4.1 Plasma α-KIC Enrichment

Plateau enrichment was attained for plasma α-ketoisocaproate, as described previously in Chapter 5, during the last 180 min of each 4 h phase of the 8 h study (Chapter 5, Figure 5.1 and Table 5.7). Also, the plasma concentration of α-ketoisocaproate was stable during each phase of the study (Chapter 5, Figure 5.3). There was a rise in plasma α-ketoisocaproate enrichment from 4.75±0.3 (4.0, 5.5) APE during Ala+Gly infusion to 6.11±0.6 (4.7, 7.5) APE during Ala-Gln infusion. However this change did not reach statistical significance (p=0.066).

7.4.2 Tracer Enrichment in Plasma Albumin

The [^{13}C]leucine enrichment in plasma albumin (Figure 7.1) increased linearly from 0.039±0.001 (0.037, 0.042) to 0.088±0.001 (0.085, 0.092), APE, during the period 60-240 min of TPN + Ala+Gly infusion ($p<0.00001$) and from 0.179±0.002 (0.0174, 0.0185) to 0.246±0.005 (0.234, 0.258), APE, during the period 60-240 min of TPN + Ala-Gln infusion ($p<0.00001$). Regression analysis of the data gave the equation for the relationship between tracer incorporation into albumin and time as $y = 0.024 x - 0.008$ during the period 60-240 min of TPN + Ala+Gly infusion (Figure 7.1 and $y = 0.033 x - 0.018$ during the period 60-240 min of TPN + Ala-Gln infusion. Extrapolation of these lines to the values for basal enrichment indicated secretion times of 20 min and 32.7 min respectively.

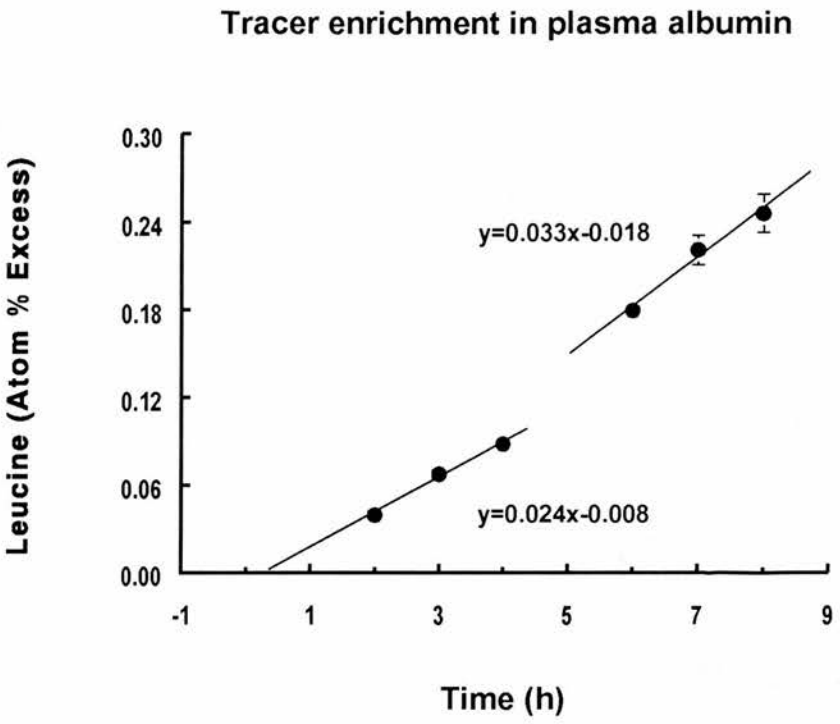

Figure 7.1 [1-^{13}C]leucine enrichment in plasma albumin during TPN supplementation with Ala+Gly (120-240 min) and during supplementation with Ala-Gln (360-480 min). The equations denote the calculated regression slopes of the curves showing linear incorporation of the tracer in each period.

Table 7.1 Calculated albumin synthetic rates assuming plasma α-ketoisocaproate (α-KIC) as precursor.

Patient	TPN + Ala+Gly (%/h)	TPN + Ala-Gln (%/h)
1	0.26	0.19
2	0.40	0.49
3	0.23	0.40
4	0.32	0.35
5	0.67	1.18
6	0.50	0.51
7	0.43	0.55
8	0.48	0.82
Mean±SEM	0.41±0.05	0.56±0.10
CI	(0.30, 0.53)	(0.30, 0.82)

7.4.3 Calculated Albumin Synthetic Rate

The calculated albumin synthesis rate was 0.41±0.05 (0.290, 0.525) %/h during Ala+Gly supplementation of TPN and was 37 % higher at 0.56±0.10 (0.304, 0.817) %/h, p=0.058, with Ala-Gln supplementation (Figure 7.2 and Table 7.1)). The calculated daily rates were 9.8±1.2 (7.0, 13.0) %/d (Ala+Gly) and 13.5±2.5 (7.3, 19.6) %/d (Ala-Gln).

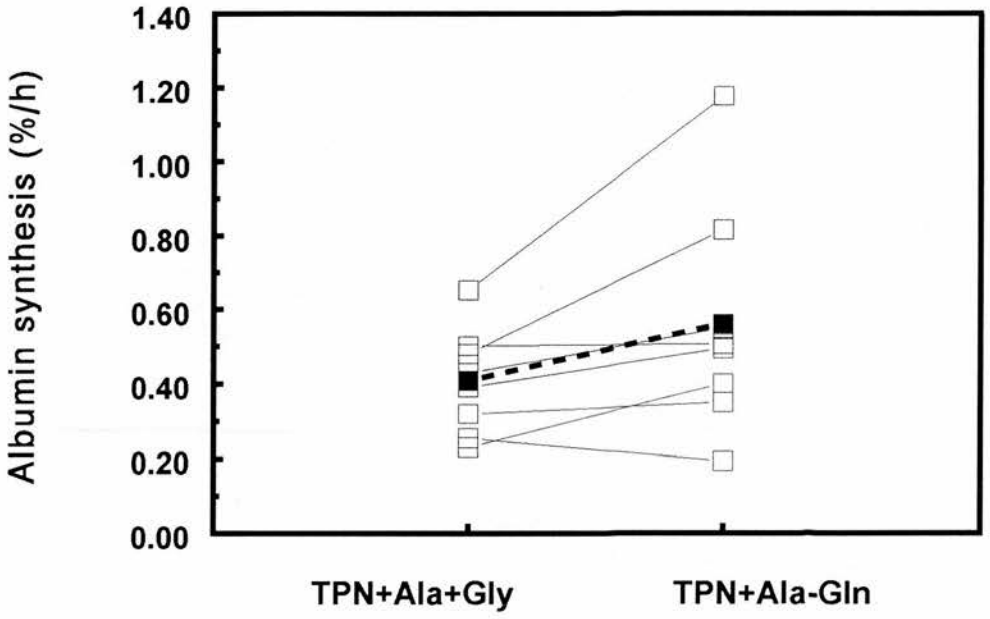

Figure 7.2 Albumin synthesis rate by [1-^{13}C]leucine incorporation, calculated using plasma α-ketoisocaproate as the precursor pool, in the longitudinal study group receiving TPN supplemented by Ala+Gly (on the left) followed by Ala-Gln (on the right). The hatched bar connects the points representing the mean synthetic rates for each period.

7.5 Discussion

Conceptually this study was a *retrospective* study and was undertaken using blood samples remaining from earlier studies investigating muscle and whole-body protein turnover; as such it may have been susceptible to the same problems of study design and execution, as well as incomplete data collection which may beset such studies and disadvantage them in comparison to prospective studies.

Nevertheless we believe the present study to be of interest, as to our knowledge, it provides the first account of an investigation into the effect of glutamine provision (as the dipeptide Ala-Gln) on albumin synthesis in man and in particular an evaluation of its role in this regard during TPN supplementation in post-surgical patients.

It is arguable that the present study's design allowed for too short a period of time, i.e. 4 h, to determine the possible acute effect of nutritional intervention. Although, in a previous study, an acute stimulatory effect of large-dose ('flooding dose') administration of the branched-chain amino acid leucine on albumin synthesis was detected over a period of 90 min (Smith *et al.*, 1993), that study was performed however on healthy young post-absorptive subjects and it cannot be assumed that liver albumin synthesis would show similar acute responsiveness to additional nutrient stimulus in older post-surgical patients receiving TPN.

Measurement of protein synthesis by stable isotope tracers ideally requires knowledge of the enrichment of the precursor pool (aminoacyl *t*RNA) from which it is being synthesised. In practice it may prove difficult or impossible to obtain tissue by biopsy sufficient to analyse this pool. Instead enrichment values of the accessible amino acid pools, which during [1-^{13}C]leucine infusion are ^{13}C-leucine or

^{13}C-αKIC, are commonly used. Although there is now a considerable body of evidence to suggest that at least for the whole-body and skeletal muscle, plasma ^{13}C-αKIC more precisely reflects and predicts the enrichment of intracellular leucine than plasma ^{13}C-leucine (Matthews *et al.*, 1982; Layman and Wolfe, 1987; Horber *et al.*, 1989), the question arises as to whether the same argument holds for more rapidly turning over proteins such as albumin. Supportive evidence to indicate an affirmative answer has come from a number of recent studies (Schwenck *et al.*, 1985; Layman and Wolfe, 1987; Pacy *et al.*, 1990).

Although a large number of studies of albumin synthesis have been reported in healthy subjects and in patients, no single study appears to provide an appropriate 'ideal' reference range to allow comparison of the values of albumin synthesis determined under the circumstances of the present study. Reported values obtained for albumin synthesis in healthy subjects show a wide range, possibly reflecting the differing investigative methods used. In healthy young subjects they range from 6.0 %/d to 12 %/d in the post-absorptive state (Smith *et al.*, 1993; Olefumi *et al.*, 1991; De Feo *et al.*, 1991). Feeding resulted in stimulation of albumin synthesis up to a value of 23 %/d (De Feo *et al.*, 1991). Also, administration of a large dose of leucine resulted in an acute stimulation of albumin synthesis from 6.0 %/d to 10.3 %/d (Smith *et al.*, 1993). Studies of albumin synthesis in patients have provided a range of values for a number of different disease conditions: 8 %/d in patients receiving a mixed solution of the branched chain amino acids alone following gastric bypass surgery for morbid obesity (Desai *et al.*, 1985); 9.6 %/d in post-absorptive patients suffering from cancer cachexia (Shaw *et al.*, 1991) and 9.9 %/d in type 1 diabetic patients in the fed state, whilst receiving replacement insulin (De Feo *et al.*, 1991). The values for

albumin synthesis observed in the present study in patients after major surgery receiving TPN, from 9.8±1.2 %/d with Ala+Gly supplementation to 13.5±2.5 %/d with Ala-Gln supplementation, fall within this broad reported range.

In conclusion, short-term glutamine provision, in the form of Ala-Gln dipeptide supplementation of TPN, appears to result in acute stimulation of albumin synthesis in post-operative patients, although in percentage terms the stimulatory effect was smaller in degree than the effect observed on skeletal muscle protein synthesis (Chapters 5 and 6). The values obtained are similar to those obtained in postabsorptive healthy volunteers (De Feo *et al.*, 1991) and therefore may represent a degree of resistance to the effects of parenteral feeding.

Following trauma, including the trauma of elective surgery, the reported fall in plasma albumin concentration may be due largely to redistribution to extravascular compartments rather than a reduction in its synthesis or increase in breakdown (Soni, 1995), although in this study we have not investigated rates of breakdown. Interventions aimed at ameliorating the pathophysiological effects of trauma or sepsis on the body as a whole are therefore more likely to be effective in restoring albumin levels to normal than interventions directed at raising albumin concentrations in plasma *per se* (Soni, 1995).

CHAPTER 8

CONCLUSIONS AND FUTURE WORK

CONTENTS

8.1 General Discussion

The studies described here are subject to a number of uncertainties and limitations. Amongst the most important is that errors may have arisen by calculation of indices of protein turnover and amino acid metabolism from the tracee enrichments in the accessible vascular compartment rather than in *t*RNA. The evidence supporting this approach in man is circumstantial. Qualitative mistakes may have arisen therefore in the present studies, particularly with overestimation of the anabolic effect of glutamine dipeptide provision on protein synthesis.

A second source of potential error, particularly in the healthy volunteer studies, has been the use of Ala+Gly as a control isonitrogenous solution against which to compare the effects of Ala-Gln on protein synthesis. The use of this solution has not yet been independently validated to have a neutral effect on protein turnover and any depressive effect it may have on protein synthesis (although no evidence for this has been reported) would lead to an overestimation of the stimulatory effect of Ala-Gln on protein synthesis. In the studies in post-operative patients, carried out at a later time, the study design was modified therefore to include the use of an 'independent' group of patients receiving TPN alone against which to compare the effects on protein synthesis of Ala+Gly and Ala-Gln supplementation.

The coefficients of variation of the measures of protein turnover were large, particularly with the use of the forearm [^{15}N]phenylalanine arteriovenous exchange method. This was due to errors compounding in the calculations based on small

arteriovenous differences and to the inter-individual variation in blood flow measurements, a major component in the calculation of protein turnover by the limb exchange method. The coefficients of variation for muscle protein synthesis calculated from tissue enrichments in samples obtained by biopsy were lower than those determined by the arteriovenous exchange technique. However, it may not be valid to directly compare the results from the two techniques as the arteriovenous exchange technique determines protein turnover at a point in time, whereas the biopsy technique gives an integrated result during a period of time. Also, in these studies different groups of limb muscles were studied by each technique, i.e. mixed muscles of the forearm *versus* the M. tibialis anterior.

It is probable that the metabolic response to surgical injury in the patients was variable, even though the nature of the surgical operation and anaesthesia was similar for all patients. In order to minimise the effects of such a variable in elucidating the effects of Ala-Gln supplementation on these patients, a further modification was introduced to the study design for one patient group by dividing the eight hour study period in two and using the patients as their own controls. In using this modified approach, however, the time scale of the protocols may not have been optimal to adequately show the effects of Ala+Gly and Ala-Gln supplementation.

Despite these qualifications, the results show that provision of the dipeptide Ala-Gln by the intravenous route resulted in significant rises in plasma and intramuscular free glutamine in healthy post-absorptive man. In comparison with a control group provided with an isonitrogenous solution of Ala+Gly, fractional muscle protein synthesis was significantly higher in the Ala-Gln subjects, as determined by [1-^{13}C]leucine incorporation into muscle protein. The rate of protein

synthesis was also higher in the Ala-Gln subjects when calculated by forearm arteriovenous exchange of [^{15}N]phenylalanine, although the difference was smaller. These findings suggest a small stimulatory effect on muscle protein synthesis of parenteral glutamine provision in dipeptide form in healthy post-absorptive man. It is possible that the stimulation in protein synthesis was mediated by the observed rise in the intramuscular free glutamine, although in the small group of subjects studied no significant positive correlation was found between the intramuscular glutamine concentration and the fractional muscle protein synthetic rate. Further studies are required on larger subject groups to validate the above findings and demonstrate conclusively whether any correlation exists in healthy man between intramuscular glutamine concentration and muscle protein synthesis, of a kind which has already previously been described in animal studies (Jepson *et al.*, 1988). Glutamine provision, as the dipeptide Ala-Gln, did not have any significant effect on the rate of skeletal muscle protein breakdown, as determined by forearm arteriovenous exchange of [^{15}N]phenylalanine, in contrast to the finding in perfused rat skeletal muscle, where it has been shown to inhibit breakdown (MacLennan *et al.*, 1988). Also, although whole-body protein breakdown, as determined by [^{13}C]leucine and [^{15}N]phenylalanine plasma kinetics, was lower in the Ala-Gln subjects in comparison with the group provided with Ala+Gly, the difference was not statistically significant.

Although the work described in chapters 3 and 4 provides for the first time information on the relationship between glutamine provision and protein synthesis (by direct measurement) and protein breakdown, respectively, in healthy man it is not possible from these studies alone to draw firm general conclusions as to what physiological role glutamine may have in modulating skeletal muscle protein

turnover and further studies to investigate the effect of glutamine provision, perhaps administered in a wider range of doses, in healthy man in the *fed* state would seem desirable to facilitate a more complete understanding.

Major surgery resulted in a significant fall in intramuscular free glutamine (> 50%), in keeping with previously reported findings (Vinnars *et al.*, 1975; Fürst *et al.*, 1984) inspite of the provision of parenteral nutrition in the form of a commercially available mixture of amino acids from the first post-operative day. It would appear therefore that conventional TPN is not capable of reversing the expected post-operative depletion of the free intramuscular glutamine pool. Also, fractional muscle protein synthetic rates remained depressed (in comparison to literature values of muscle protein synthesis rates in healthy man) when patients were studied on their third post-operative day.

Ala-Gln supplementation of conventional TPN in post-operative patients resulted in significant rises in the plasma and intramuscular free glutamine concentrations, in comparison (within the same group of patients) to supplementation of TPN by an isonitrogenous control solution of Ala+Gly and also in comparison to a separate group of patients provided conventional TPN alone. Ala-Gln supplementation also resulted in a significant rise in the fractional muscle protein synthetic rate, as determined by [1-^{13}C]leucine incorporation into tibialis anterior muscle protein, regardless of calculation based on two different surrogate precursor pools for leucyl-*t*RNA: α-ketoisocaproate and intramuscular free leucine. The rate of protein synthesis was also significantly higher with Ala-Gln supplementation when calculated by forearm arteriovenous exchange of [^{15}N]phenylalanine, although the difference was smaller. In absolute terms, the rates of muscle protein synthesis in this group of parenterally *fed* patients only just

approached the values observed for muscle protein synthesis in the post-absorptive healthy volunteers. This latter comparison can only be made with great care as there are many other variables separating the two groups, including age (patients were older) and pre-study general state of health (patients suffered from oesophageal carcinoma). Nevertheless the present study appears to demonstrate that although glutamine dipeptide supplementation of conventional TPN in patients undergoing major surgery partly repletes their intramuscular glutamine pool and raises the muscle protein synthetic rate, there may be some residual 'resistance' to a return to normal muscle protein homoeostasis inspite of what would appear to be adequate nitrogen and energy provision.

8.2 Future work

The results of glutamine provision as Ala-Gln in healthy subjects are intriguing and raise questions regarding the physiological basis for glutamine's role in modulating muscle protein balance in health in man. Further work, at both cellular and tissue level, needs to be undertaken to elucidate the mechanism(s) by which glutamine stimulates muscle protein synthesis.

With respect to post-operative patients, in the present studies information on whole-body breakdown only was obtained as these particular patients were unable to tolerate our method for collection of expired breath by Douglas bag. Further studies need to be undertaken therefore to investigate the effect of Ala-Gln on whole-body protein turnover, using commercially available portable 'metabolic carts' to measure CO_2 output accurately. In order to answer the question as to

whether improving muscle protein balance by glutamine provision is associated with concomitant improvement in post-operative skeletal muscle strength/function, it would be desirable to study this separately, perhaps as part of a prospective randomised, controlled metabolic and clinical study (see below) and establish if there is any correlation between improved muscle protein balance and muscle strength/function, as determined for example by dynamometry (Jeejeebhoy, 1986) and electromyographic studies as well as respiratory function tests.

8.3 Clinical Implications

Recently, in growing recognition of the important role of glutamine in the nutritional care of the critically ill, a number of commercially available glutamine-enriched products for *enteral* feeding have become available (e.g. Alitraq, Ross Products Division, Abbott Laboratories, England). Based on the findings of this work, considering alone the potential benefits to skeletal muscle physiology in the post-operative patient, it would seem that there is some evidence to support the routine inclusion of glutamine or a glutamine dipeptide such as Ala-Gln in commercial TPN solutions. However before this approach can be advocated, a number of other questions regarding glutamine supplementation of TPN, such as its effect on the immune system, would need to be answered. O'Riordain and colleagues (O'Riordain *et al.*, 1994), have recently investigated the role of glutamine supplemented TPN on T-lymphocyte response in surgical patients undergoing colorectal surgery. There was a significant rise in T-cell DNA synthesis ($p<0.05$) over the pre-operative values with glycyl-glutamine dipeptide supplementation in comparison to the group receiving conventional TPN. Interestingly, in contrast to

earlier investigators, they additionally found no significant improvement in nitrogen balance with glutamine supplementation. Pre- and post-operative plasma and intra-muscular free glutamine concentrations as well as tissue and whole-body protein turnover were not studied and patients were not assigned injury scores. Nevertheless this study highlights the need for exercising caution in extrapolating the findings of earlier studies on the effect of glutamine supplementation on nitrogen balance to all catabolic patients regardless of the severity of the 'injury'. It is possible that in this study, as the authors have suggested, the patients were only mildly catabolic and glutamine supplementation may not have been able to further improve their nitrogen economy as they were already in neutral nitrogen balance. Petersson and colleagues (Petersson *et al.*, 1994) have also recently reported a similar finding of a failure of glutamine supplemented TPN in altering whole-body nitrogen economy in post-operative patients undergoing elective (open) cholecystectomy, although the post-operative decline in muscle protein synthesis (as assessed indirectly by total ribosome concentration) observed in the control group did not occur in the glutamine supplementation group.

In a wider context, using prospective randomised controlled clinical trials with larger cohorts of patients, parameters such as hospital stay, complication rates (infection, respiratory, delayed wound healing) and overall mortality and morbidity would need to be investigated in detail to assess the likely cost-benefits of glutamine supplementation. Such a long-term study is being undertaken by Griffiths and colleagues on intensive care patients (Rennie, 1993). It is possible that such general clinical studies, with a much broader outlook than allowed by the limited scope (essentially biochemical/metabolic) of the present work, may also reveal unexpected and untoward 'side-effects' of glutamine supplementation.

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